H
01
782
B10
UNIVERSITY
OF MICHIGAN
JAN 22 1952
SCIENCE
LIBRARY

VOLUME 27 NUMBER 1

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

JANUARY 1952

CONTENTS

Water-Pretreatment Squash Technic. SAJIRO MAKINO and ISAO NISHIMURA..... 1

Demonstration of Golgi Zones by Three Technics for Carbohydrates. J. P. ARZAC and L. G. FLORES..... 9

Staining Methods for Studying Ingredient Distribution in Baked Cakes. MARTHA E. JOOSTE and ANDREA OVERMAN MACKEY..... 17

Paper Chromatography for Analysis of a Dye Mixture. ARTHUR J. EMERY, JR. and ELMER STOTZ..... 21

Rapid Preparation of Thick Sections of Fleshy Plant Tissues. R. M. REEVE..... 29

Ethylenic Reaction of Ceroid with Performic Acid and Schiff Reagent. R. D. LILLIE.. 37

A Direct Silver Method for the Golgi Apparatus. HERBERT ELFTMAN..... 47

Chromatography and Biological Stains. JOHN F. CHRISTMAN and ROBERT H. TRUBEY.. 53

Differentiation of Two Types of Basophils in the Adenohypophysis of the Rat and the Mouse. NICHOLAS S. HALMI..... 61

OFFICIAL ORGAN OF THE BIOLOGICAL STAIN COMMISSION

Published by BIOTECH PUBLICATIONS, Geneva, New York

Entered as second class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

ISSUED BIMONTHLY

SUBSCRIPTION PRICE $5.50 PER YEAR (U. S. CURRENCY)

Editor and Business Manager, H. J. CONN, Geneva, N. Y.
Associate Editor: H. A. DAVENPORT, Northwestern Univ. Med. School
Assistant Editor, JEAN E. CONN, Middletown, Ct.

Consulting Editors

ABSTRACTS: G. H. CHAPMAN, New York, N. Y.
ZOOLOGY: S. I. KORNHAUSER, School of Medicine, Univ. of Louisville
PATHOLOGY: R. D. LILLIE, National Inst. of Health
BOTANY: CONWAY ZIRKLE, Univ. of Pennsylvania

Publication Committee of Biological Stain Commission

LOUIS GERSHENFELD, Chairman; H. A. DAVENPORT; E. H. STOTZ

SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Box 269, Geneva, N. Y.

Articles dealing with microscopic technic, or with the nature or uses of biological stains are suitable for publication in STAIN TECHNOLOGY. It is understood that articles submitted have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which must be complete in itself and contain all the important facts brought out in the paper. When possible the abstract should summarize the new methods in such form that a reader can follow the directions without consulting the body of the paper.

All staining formulae must be definite and accurate. The batch or certification number of the stains actually used by the author in such formulae should be included for reference purposes. In case of uncertified stains, not designated by any batch number, one should give as much information as available, i. e. name of manufacturer, approximate date of purchase, etc.

In general all copy should be typewritten, either double or triple spaced; although complicated material such as long tables is acceptable if written or printed legibly by hand. Legends for tables should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. All references to literature must be carefully verified and ordinarily the complete title and reference should be given

All illustrations should accompany the manuscript and should be numbered, with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. *Colored illustrations are accepted only at the author's expense.*

STAIN TECHNOLOGY

VOLUME 27 JANUARY 1952 NUMBER 1

WATER-PRETREATMENT SQUASH TECHNIC

A NEW AND SIMPLE PRACTICAL METHOD FOR THE CHROMOSOME STUDY OF ANIMALS[1]

SAJIRO MAKINO and ISAO NISHIMURA, *Zoological Institute, Hokkaido University*

Received for publication Jan. 8, 1951

ABSTRACT.—**To simplify the staining of animal chromosomes (especially in insect testes) the authors have borrowed (with necessary modifications) the squash technic of plant cytology. The method has four steps: (1) *Water pretreatment.* This step requires only about 5–10 minutes either in water at room temperature or in water kept at about 38°C. in a water bath. (2) *Fixation.* Ordinarily only 5 minutes in 10–15% aqueous solution of glacial acetic acid is necessary. (3) *Staining.* The fixed tissue is rinsed in two or three changes of distilled water and then placed in a solution of basic fuchsin: either 1% in 30% ethyl alcohol, or 0.2–0.4% in 5–10% lactic acid. In the former solution the staining period should be about 2 minutes: in the latter, 5–20 minutes. The time is not critical. (4) *Squashing.* The material is rinsed in several changes of distilled water, placed on a clean slide and squashed under a cover glass. Such preparations last 4–5 weeks, and a technic is described for removing the cover glass in order to mount in Euparal and to make them permanent. The authors list various species of vertebrates as well as invertebrates in which the technic has given good chromosome staining, as shown by illustrations.**

Much of past work on animal chromosomes, especially in the case of the chromosomes of the higher vertebrates, has been done on sectioned material fixed with various kinds of fixatives that contain a costly reagent, osmic acid. Flemming's chromo-osmic solution and Champy's chromo-osmic-bichromate mixture are examples of such fixatives. In practice, the classical paraffin method is very troublesome to apply, requires a considerable length of time, and has many shortcomings in the results. Though a surprising advance has been made in the chromosome study of animals based on this method,

[1]Contribution No. 241 from the Zoological Institute, Faculty of Science, Hokkaïdo University, Sapporo, Japan. Aided by the Scientific Research Fund from the Ministry of Education.

there seems to be little prospect for further progress. A new approach to the problems involved is through the application of the squash or smear technic, borrowed from plant cytology. While working with the spiral structure of grasshopper chromosomes by the use of a new acetocarmine technic (Makino and Momma, 1950, J. Morph., **86**), the senior author (Makino) obtained the impression that this technic for the study of chromosome structure may be applicable also for the preservation of chromosomes themselves. Thus, repeated attempts which were made with the hope of modifying the method for use in the preservation of chromosomes of many kinds of animals afforded success in developing a new technic. This consists of the combination of pretreatment with water, acetic fixation, and fuchsin staining. It is capable of giving good results for studying the chromosomes of many animals, especially for studying their number, morphology, and behavior.

Technic

The technic on which the present report is being made consists of four successive procedures: (1) water-pretreatment, (2) acetic fixation, (3) fuchsin staining, and (4) squashing. The reagents necessary for the technic are distilled water, a 10–50% acetic acid solution, and a fuchsin solution. Testicular tissue of various animals is used as test material.

Pretreatment with water. In insects[2] and other invertebrates, the testes are removed by vivisection and then submerged, *without squashing and pre-fixing,* either in tap water or in distilled water in a clean glass vessel of proper size. Previous to immersion, it is necessary to remove the fatty tissue carefully as well as any other tissues adhering to the testis. In insects, the testes can be treated *in toto,* but in vertebrates they should be cut into several pieces before the pretreatment. The testes may be left in water from 5 to 10 minutes at room temperature. In treating the testicular material of birds, immersion in warm water kept at approximately 38°C. by the aid of a water bath, gives good results. The time of pretreatment does not seem to affect the results materially, about a 5-minute immersion being sufficient in most cases, both in invertebrates and in vertebrates.

Also, the pH of the water used for pretreatment does not seem to influence the results much, since every case of pretreatments with tap water of pH 7.4 and pH 5.0, and distilled water of pH 5.5 showed nearly equal results. The same seems to be the case with the water temperature; the variation ranging from 11°C. to 15°C. shows no noticeable difference in result.

[2]In the case of the ovarial tissue of insects, so far as the treatments on some locusts and crickets are concerned, the pretreatment with 0.25–0.5% salt solution for about 5 minutes seems to be appropriate, although no uniform result is obtainable.

Fixation. After water pretreatment, the testes are transferred to a vial containing a fixing solution, in which they are left for about 5 minutes. As the fixing reagent, a 10–50% aqueous solution of glacial acetic acid is recommended. Other fixatives were tried (Bouin, Carnoy, and Nawashin, etc.), but good results were obtained only with a single solution of acetic acid. In this case the fixation leaves the tissue so soft that the cells can be separated by simple mashing. Different concentrations of acetic acid seemed to have no specific effect on the results, since 3 and 5% acetic acid proved equally satisfactory.

Fixation for 5 minutes was found to be enough in most cases, both in the invertebrates and in the vertebrates so far examined. However, the time of fixation calls for special precautions. Treatment for too long a time makes the tissue too hard or too tough to be mashed out.[3] Strictly speaking, the length of fixation time differs in different material. The most suitable time can be learned only by experience.

When necessary, the material can be stored in 5–10% acetic acid after fixation.[3]

Staining. After fixation, the tissue is rinsed in distilled water with two or three changes, of 2–3 minutes each. Then the tissue is placed into a small vial containing either of the two following staining solutions:

1% aqueous basic fuchsin: Dissolve 1 g. of basic fuchsin in 30 ml. of hot 95% alcohol, and then add 70 ml. of distilled water. Shake well and filter.

Lactic acid solution of basic fuchsin: Dissolve 0.2–0.4 g. of basic fuchsin in 100 ml. of 5–10% lactic acid solution. Shake well and filter.

In case the 1% aqueous fuchsin solution is used, staining requires about 2 minutes. The degree of staining depends more or less on the size of the tissue block. Sometimes only one minute is sufficient when the testis pieces are of a small size. It should be remembered that over-staining induces intense coloration of the cytoplasm which prevents a sharp differentiation of the chromosomes and, at the same time, makes the material tough.

In the case of lactic acid solution of fuchsin, the time of staining does not seem to influence the results to any noticeable extent, since staining either for 5 minutes or 20 minutes gave about the same results. However, this stain generally gives less sharp differentiation of the chromosomes from the cytoplasm than does the use of 1% aqueous fuchsin.

[3] A longer fixation or storage makes the material tough; in this case the material should be immersed in a solution consisting of HCl, 90% alcohol, and distilled water in the proportion of 1:1:1, for 5 to 10 minutes, and then rinsed in water.

Staining is good just after acetic acid fixation, but it becomes increasingly more difficult after storage in acetic acid.

Squashing. After staining, the material is rinsed in several changes of distilled water for a few minutes. To prepare the stained material for microscopic examination, it is placed on a clean slide and covered with a cover glass. Complete spreading of the cells may be accomplished by rubbing the surface of cover glass by means of a blunt needle. Where necessary, a flat plate of material may be obtained by carefully applying an even pressure on the cover glass under several layers of blotting paper. To seal the cover glass an improved sealing medium[4] should be used. The preparation is then ready for study. These temporary preparations, when completely sealed, will keep from 4 to 5 weeks without further deterioration if kept in a cool dark place.

The temporary slide can be made permanent by observing the following procedures:

For permanent preparation, staining with a lactic acid solution of fuchsin gives good results. The stained material is placed on an albuminized slide. It is necessary to place a piece of moistened cellophane of adequate size between the material and the cover glass. An even pressure is applied on the cover glass under several thicknesses of blotting paper. The slide thus prepared is then placed in a tall jar containing distilled water. After a few minutes both the cover glass and the cellophane become separated from the slide and the material remains fixed to the albuminized slide. Then the slide is rinsed well in distilled water, dehydrated with an increasing alcohol series, mounted in Euparal from 95% alcohol without transferring to xylene, and covered with a cover slip.

In miscroscopical examination, it is desirable to use a green filter.

Results

The technic presented above was tried on testes of 32 species of invertebrates and 16 species of vertebrates. In every case examined, the material showed uniformly satisfactory results in the demonstration of both mitotic and meiotic chromosomes. The species of animals examined are listed in Table 1. The results obtained probably may be appreciated more precisely by referring to the accompanying figures than by any descriptive explanation (Fig. 1–17). To the regret of the authors, the results were unsuccessful in the case of the fishes so far examined, i.e., the loach and goldfish.

---

[4]This medium consists of nearly equal parts of hard paraffin and Canada balsam, melted together by gentle heat. This forms a solid mass on cooling. For applying to the cover glass a heated spatula is used.

TABLE 1. LIST OF ANIMALS TO WHICH THE WATER-PRETREATMENT SMEAR TECHNIC WAS APPLIED WITH SATISFACTORY RESULTS.

INVERTEBRATA

INSECTA

Orthoptera: *Podisma sapporoense, Miramella mikado, Acridium japonica, Parapreurus alliaceus, Oxya ezoensis, O. velox, Metrioptera japonica, Gampsocleis mikado, Homorocoryphus jezoensis, Tettigonia orientalis, Gryllus mitratus, Scapipedus parvus, Diestrammena japonica.*

Lepidoptera: *Bombyx mori, Lymantria dispar, Dendrolimus spectabilis.*

Odonata: *Sympetrum frequense, Mnais costalis.*

Neuroptera: *Chrysopa japana, Ch. sapporoensis.*

Hemiptera: *Dolycoris baccarum, Spilostethus equestris, Eusarcoris ventralis.*

Homoptera: *Aphrophora costalis.*

Coleoptera: *Epilachna vigintioctmaculata, Coccinella bruckii, Popillia japonica, Cicindela* sp., *Anisodactylus* sp.

CRUSTACEA: *Eriocheir japonica, Xiphocaridina compressa.*

PULMONATA: *Succinea lauta.*

VERTEBRATA

AMPHIBIA: *Rana temporaria, R. nigromaculata, R. japonica, Hyla arborea. Triturus pyrrhogaster.*

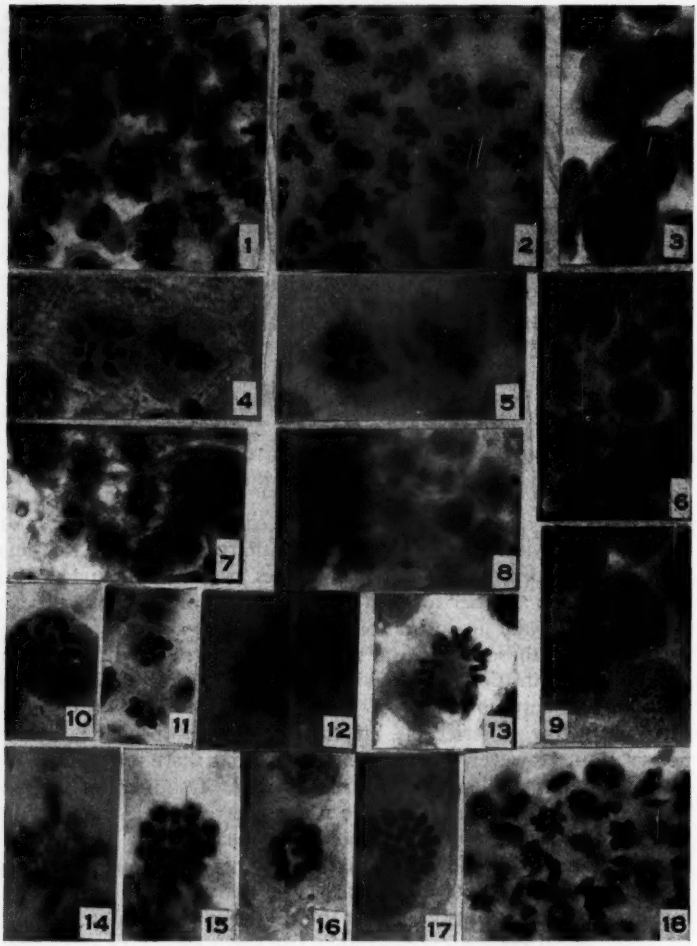
REPTILIA: *Tachydromus takydromoides, Amyda japonica.*

AVES: *Passer montanus, Gallus gallus dom.*

MAMMALIA: *Mus molossinus, Mus musculus, Rattus norvegicus, Clethrionomys mikado, Lepus cuniculus, Cricetus* sp., *Bos taurus.*

In general, by the application of this technic, the chromosomes at metaphase are visible with a well-defined, sharp outline, and extreme clearness of appearance. The chromosomes are found arranged well apart from one another to form a regular equatorial plate; there is no sign of irregular agglutination or clumping of chromosomes even in the case of those of minute sizes, nor is there shown any other obscure configurations which would make accurate observation difficult. The chromosomes show a tendency to be slightly thickened, often with clear constrictions, but this presents no obstacle at all for observation. However, it should be added that there are some cases in which better results are obtainable by direct treatment of the material with acetocarmine after the water pretreatment, than by the successive treatment with acetic acid and fuchsin. For example, in *Podisma,* and in some dragonflies, the chromosomes appear in more clearly defined outline with acetocarmine than with fuchsin. In the smear preparations thus prepared, the counting of chromosomes and the study of chromosome morphology and behavior can be made with accuracy and ease. The large size of the squashed cells assists the observation also.

The technic here presented has several advantages, of which the most important are its simplicity, speed of procedure, and the excellent results both in preservation of chromosomes and in their staining. Both cells and chromosomes are strikingly free from shrinking — a condition generally encountered in paraffin sections. Consequently, the chromosomes prepared with this technic are surprisingly larger in size than are those in sections. Another advantage is that no expensive agent, such as osmic acid, needs to be used.



FIGS. 1–17. Photomicrographs of chromosomes of various animals which were fixed and stained according to water-pretreatment technic.

1, *Metrioptera japonica,* spermatogonial metaphases, × 400. 2, the same I-metaphases, × 400. 3, *Acridium japonica,* I-metaphases, × 400. 4, *Podisma mikado,* I-metaphase, × 400. 5, *Homorocoryphus jezoensis,* I-metaphase, × 600. 6, *Eusarcoris ventralis,* I-metaphases, × 600. 7, *Coccinella bruckii,* I-metaphases, × 600. 8, *Popillia japonica,* I-metaphases, × 600. 9, *Bombyx mori,* I-metaphases, × 600. 10, *Succinea lauta,* I-prometaphase, × 600. 11, *Tachdromus takydromoides,* I-metaphase, × 600. 12, *Hyla arborea,* I-metaphase, × 1200. 13, *Rana temporalia, spermatogonial* metaphase, × 600. 14, *Gallus gallus dom.,* I-metaphase, × 1500. 15, *Mus musculus,* I-metaphase, × 1000. 16, the same, II-metaphase, × 1000. 17, *Lepus cuniculus,* I-metaphase, × 1200. 18, Spermatocyte chromosomes of *Mus musculus,* from paraffin section of testis which was pretreated with water and fixed in 50% acetic acid, × 600.

An attempt was made to transfer the pretreated, fixed material to the paraffin method. When pieces of the mouse testis, which were fixed with 50% acetic acid after water-pretreatment, were transferred to the paraffin method, the sections stained with Heidenhain's iron-hematoxylin showed metaphase chromosomes in a state of good preservation, although there was a considerable shrinkage of the cell (cf. Fig. 18).

Some supplementary experiments were made to observe the effect of water-pretreatment, with the application of solutions other than water in different concentrations, using grasshopper germ cells as the material. The results of these experiments seemed to show that hypotonicity of the test media was less harmful on the preservation of chromosomes than hypertonicity. It seems to the authors that the effect of water-pretreatment is regarded as a display of phenomena associated with hydration and dehydration in the cell (Kuwada, 1937, Cytologia, Fujii-Jubilee Vol.). A tentative interpretation for the effect of water-pretreatment is proposed as follows: The cytoplasm is first hydrated to a certain degree. Then the spindle area is supplied with water from the hydrated cytoplasm, which results in a swelling of the substance surrounding chromosomes. The swollen substance causes the chromosomes to be well scattered and evenly distributed on the equatorial plate. In this state the cells are fixed with acetic acid.

The authors wish to acknowledge their cordial gratitude to Emeritus Professors K. Oguma and Y. Kuwada of Hokkaido University for valuable suggestions. Sincere obligation is also due to Professor W. H. Leonard of the Colorado A. & M. College, Ft. Collins, Colorado, for his kind aid in the publication of this paper.

DEMONSTRATION OF GOLGI ZONES BY THREE TECHNICS FOR CARBOHYDRATES

J. P. Arzac and L. G. Flores, *Laboratorio de Endocrinologia e Investigacion Medica, Hospital Español, Mexico, D. F.*

Received for publication May 5, 1951

Abstract.—**The carbohydrate of the Golgi apparatus of several organs of rats, rabbits, and frogs was selected as the principal test material for the behavior of three different technics: 1) periodic acid with colored fuchsin; 2) "direct" chromic acid piperazine silver; 3) periodic acid with leucofuchsin.**

Parallel sections of organs in which positive reactions were observed, were treated before staining with a series of reagents to characterize them as glycoprotein.

The results obtained by the three technics under any constant set of conditions were essentially identical in all cases. It is concluded that discrepancies that may have been noted up to now are due to several factors, probably the most important being the tissue's physiological status and the influence of fixation. The study shows that HIO_4-fuchsin[1] and chromic acid silver methods are, at least empirically, as valid as HIO_4-leucofuchsin technics.

Considering the differences in the oxidative mechanism of chromic and periodic acids and other data, the possibility of two different chemical pathways leading to the same final result is discussed.

It has been found that colored fuchsin, as well as its leuco form, can be used in the histochemical demonstration of aldehydes after periodic acid treatment (Arzac, 1948). In a later report (Arzac, 1950), a series of reactions were obtained with colored fuchsin which differed in several ways from the results of others using Hotchkiss' method. For example, Gersh (1949) reported the presence of probable glycoproteic granules in the Golgi apparatus of rabbit and guinea pig's intestine. Leblond (1950) also found positive Golgi reactions in different cells of male excretory ducts and in other organs of the rat. Such reactions had not been observed with the colored fuchsin technic in any of the two above-mentioned occasions. Since the latter investigators used different fixatives, which might have caused the discrepancies, the experiment described below was undertaken to study: (a) the influence of fixation on the final reactions elicited by HIO_4-fuchsin (colored and leuco-form) and chromic acid piperazine silver methods; (b) the results obtained in the demonstration of Golgi zones of several rat's, rabbit's and frog's organs by these methods.

[1]Whenever the "HIO_4-fuchsin" or "periodic acid fuchsin" technic is mentioned here, the use of *undecolorized* fuchsin is to be understood.

Experimental

Thirty four rats, seven rabbits, and seven frogs were the source of our material. Their organs were fixed in 96% alcohol, 10% alcoholic formalin with and without acetic acid, 10% formalin, Orth's fluid, with and without 5% acetic acid, and dioxane saturated with picric acid. The tissues were embedded in paraffin as usual and cut at 6–8 μ. Samples of analogous tissues of our previous series fixed in alcohol, alcohol-formol, and picro-alcohol-formol (with and without acetic acid) served for comparison.

Table 1. Effect of Fixation on the Reactivity† of Golgi Zones and Other Structures of Two Animal Organs

Fixative	Intestine					Testicle	
	Species	Golgi Zone	Muscular reticulum	Striated Border	Mucine	Head-caps	Acro-some
Alcohol	Rat	0	±	2	4	±	0
	Frog	1*	3	2	4		
Alcohol-Formol	Rat	3	2	4	4	2	±
	Frog	2	3	3	4		
Alcohol-formol-acetic	Rat					2	1
	Frog	4	3	4	4		
Picro-alcohol-formol	Rat	1	2	4	4	1	1
	Frog	3					
Bouin	Rat	2	3	4	4	3	3
	Frog	4**	4	4	4		
Orth	Rabbit	2	2	3	4		
	Rat	2	3	4	4	¾	4
	Frog	4	4	4	4		
Orth-acetic	Rat	1	2	4	4	3	4
	Frog	2	1	4	4		

†Periodic acid-fuchsin reaction.
*Degrees of intensity and/or completeness of the reaction.
**Diffuse.

The methods used were HIO_4-fuchsin (Arzac 1950),[2] HIO_4-Schiff according to Lillie (1947) and piperazine-silver (without formol reduction) after oxidation with chromic acid, according to Arzac and Flores (1948)[3]. In all instances digestion with saliva and hot chloroform-methanol extraction were done in control slides of the same tissue. Also the oxidative steps of the technics were omitted in other tissue sections.

[2]Eventually 1% HIO_4 oxidation was increased to 10 minutes and acid alcohol extraction reduced to one or two minutes to accentuate reticulum.

[3]One to 1½ hour staining at 50°C. in the following mixture: piperazine, 0.33 g.; $AgNO_3$, 0.10 g.; Na_2CO_3 (sat. aq.), 20 drops; distilled water, 100 ml.

The organs studied were the following: testicle, ductuli efferentis, ductus deferens, prostate, seminal vesicles, ovary, Fallopian tubes, uterus, placenta, salivary glands, stomach, intestine, hypophysis, thyroid, adrenal, kidney, cerebrum and medulla. However, attention was concentrated mainly upon rat's male reproductive organs and frog's intestine.

All the following photomicrographs were taken from organs fixed in Orth's fluid. Excepting when indicated, no counterstain was used. Fuchsin reactions were photographed through a Wratten green filter.

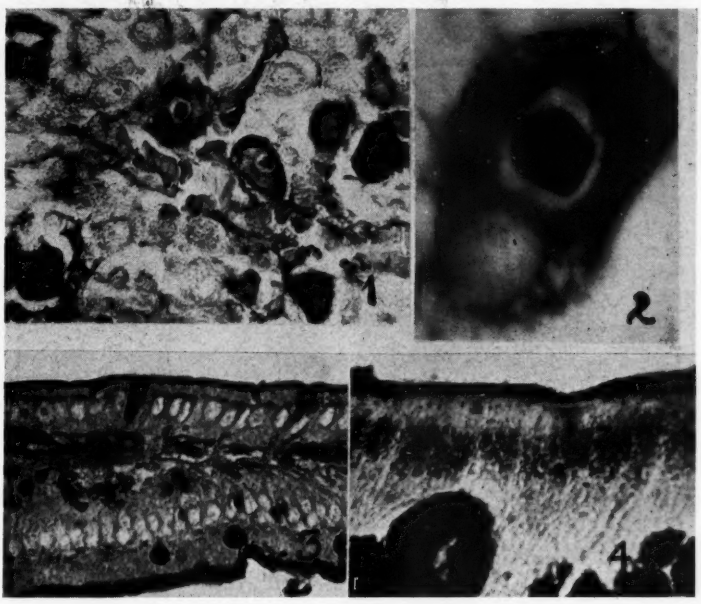


FIG. 1. — Rat's hypophysis. (Reactive) granules and Golgi apparatus of basophilic cells are seen. Periodic acid fuchsin technic.

FIG. 2. — (High magnification) of a basophilic cell. Chromic acid piperazine silver "direct" technic.

FIG. 3. — Rat's intestine after 48-hour fast. Golgi apparatus, goblet cell's mucine and striated border react positively. Not counterstained. Magnification about × 400.

FIG. 4. — Frog's intestine after 5-day fast. The abundance and intensity of reacting granules of the Golgi zone were outstanding in this case. Mucine of goblet cells may be seen below. The striated border also reacted very intensely. Chromic acid piperazine silver. About × 1,800.

RESULTS

Effect of fixation. Table 1 shows rather typical results in two of the organs studied. It can be seen that marked differences of stainability were clearly observed after one or the other fixatives. With

the exception of glycogen (which was better preserved in alcoholic mixtures) all the other substances and structures were more intensely and sharply stained in tissues fixed in dichromate-formol. In no instance were satisfactory "Golgi positive reactions" observed in rat or rabbit tissues fixed in alcohol or alcoholic mixtures lacking

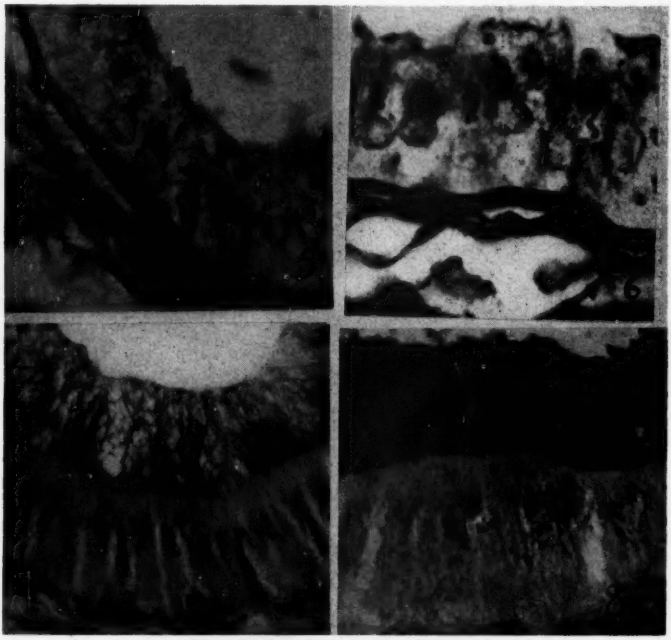


FIG. 5. — Rat's ductulus efferens. Periodic acid fuchsin without counterstain. Golgi zones' reactive material is clearly seen. Note basal membrane. About × 1,200.

FIG. 6. — Another section of same organ stained with chromic acid piperazine silver "direct" method. Golgi reactions are also sharply demonstrated. Here too, note basal membrane and reticulum. Not counterstained. About × 1,800.

FIG. 7. — Rat's deferens stained with periodic acid fuchsin technic. Granulues in cytoplasm and Golgi zone are intensely stained. Note sharply defined stereocilia. About × 400.

FIG. 8. — Rat's deferens. Collagen reacts intensively (without the need of formol reduction). Chromic acid piperazine "direct" method.

formol. Picric acid added little or no advantage to the alcohol in the absence of formol. Only Orth's fluid (and, though not as well, formol in the case of hypophysis) permitted a sharp demonstration of reactive substances in the Golgi zones. The addition of acetic acid to any of the above mentioned fixatives did not seem to improve the results.

The stability conferred by Orth's fluid to the reactive substances in Golgi zones was outstanding. The energetic proteolytic digestion with pepsin was practically the only treatment that had a detectable effect on them.

Reactive substances and Golgi zones observed. To avoid a need-

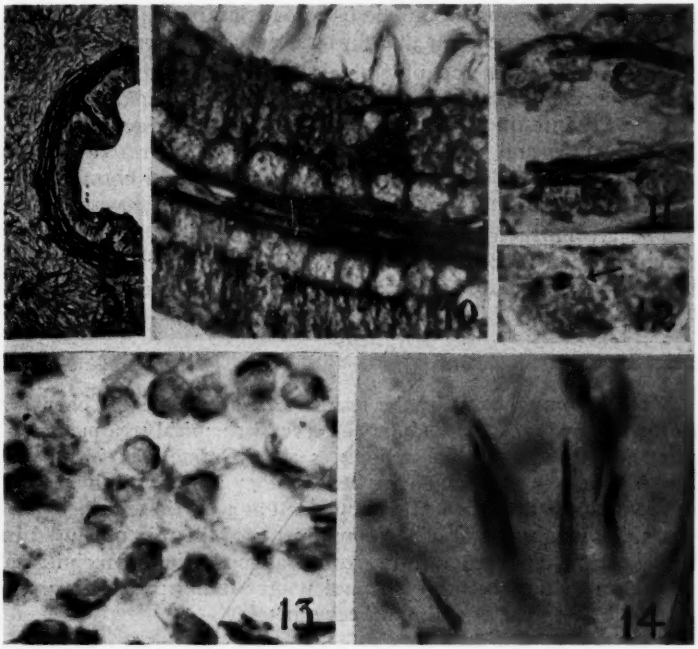


FIG. 9. — Section of same organ stained with chromic acid piperazine silver. Same distribution of the reaction is seen but stereocilia and collagen show a heavier rendering.
FIG. 10. — Epididymis of rat. Periodic acid fuchsin. Counterstained with light green. About × 1,200.
FIG. 11. — Golgi apparatus of spermatic cells. Chromic acid piperazine silver. About × 1,000.
FIG. 12. — High magnification of a similar cell. Note that the reactive material appears as a sharply defined circle.
FIG. 13. — Spermatids' caps stained with the periodic acid fuchsin procedure.
FIG. 14. — Acrosome and head cap of spermatozoa. Chromic acid piperazine silver. About × 1,200.

less repetition of descriptions, it can be stated that all the reactions and locations previously reported by one of us (Arzac 1948, 1950) on tissues fixed in alcoholic fluids plus those found by Leblond (1950) after Orth's fixation were confirmed in this study. Moreover, other locations of positive reactions can be observed, like those in the Golgi

zones of rat's and rabbit's corpus luteum cells and of frog's intestine.

When using the three methods after a given fixative, the resulting reactions were identical in quality and very similar in degree or intensity. Thus both fuchsin technics always gave practically identical results, but showed somewhat different degrees of intensity compared with those elicited by the silver method. Golgi zones, caps of spermatids and acrosome of spermatozoa were much more sharply demonstrated by the silver than by either of the other two methods.

Species differences and physiological status differences were evident. For example, after fasting both animals, frog's *(Rana Montezuma* Bair) intestine was the more constant and richer in reactive Golgi granules and rabbit's the poorer. No positive reactions were observed when oxidation with either periodic acid or chromic acid was omitted prior to staining.

The accompanying photomicrographs show results in different organs stained with different technics. To emphasize the identity between many fuchsin and silver reactions most of them have been duplicated.

Comment

The fact brought out by the results of fixation and the behavior of the reactive substances under the several chemical treatments applied is that the reactive substances are in some way intimately related to tissue protein, either because they are chemically bound to protein or because protein is important as a physico-chemical substrate. Although several of the components of the mixtures used in this experiment are generally recognized as good protein precipitants, they have in the absence of formol poorer fixing properties upon protein tissue than formol and heavy metals.

Some explanation had to be provided for the surprising similarity of the reactions obtained by the three methods used. The results observed after fuchsin or leucofuchsin may be attributed to chemical reactions between these substances and the aldehydes resulting from the oxidation of 1–2 glycol groups or amino-alcohol groups with periodic acid (Jackson and Hudson, 1938; Hotchkiss, 1948). This explanation, however, does not hold for the third method, (chromic acid silver technic) since chromic acid cannot replace periodic acid as an oxidizing agent prior to fuchsin staining. Another possible explanation may be based on the data of Smith (1946) who showed that, in carbohydrates, "preferential oxidation of the secondary alcoholic group adjacent to the carboxyl group...can be carried out by chromic acid". Such secondary alcoholic groups would have to be present in the molecules holding the periodic acid-reactive groupings. Thus, the identical results would be produced through different pathways.

Summary

A component, presumably consisting of carbohydrate material, of Golgi zones of rat's, rabbit's and frog's organs has been demonstrated by three different histochemical methods after fixation in Orth's fluid.

In all instances the simpler HIO_4-carbolfuchsin technic gave identical results with those elicited by the more elaborate HIO_4-Schiff method, with which the substances in question were first demonstrated by other authors.

After chromic acid oxidation, piperazine silver complexes always showed closely similar if not identical distribution in the same tissues.

The results obtained with the three technics always varied in a parallel way according to the fixative used. Thus, Golgi zones never appeared or were poorly demonstrated after defective tissue protein fixatives. It was clearly evident that differences of species and physiological status caused variations of reactivity.

Acknowledgements

We acknowledge with pleasure the technical assistance of Miss Ofelia Cortina.

Literature Cited

Arzac, J. P. 1948. Acido peryódico carbolfuchina para la demostración de carbohydratos en los tejidos. An. Méd., **9,** 15–17.

——— 1949. Acidos crómico y peryódico en la demostración argéntica del glucógeno. Rev. Mex. Lab. Clínico, **2,** 107–13.

——— 1950. A simple histochemical reaction for aldehydes. Stain Techn. **25,** 187–94.

Arzac, J. P., and Flores, L. G. 1949. The histochemical demonstration of glycogen by silver complexes. Stain Techn., **24,** 25–31.

Gersh, I. 1949. A protein component of the Golgi apparatus. Arch. Path., **47,** 99–109.

Hotchkiss, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., **16,** 131–41.

Jackson, E. L., and Hudson, S. C. 1938. The structure of the products of the periodic acid oxidation of starch and cellulose. J. Amer. Chem. Soc., **60,** 989.

Leblond, C. P. 1950. Distribution of periodic acid reactive carbohydrates in the adult rat. Amer. J. Anat., **86,** 1–25.

Lillie, R. D. 1947. Studies on the preservation and histologic demonstration of glycogen. J. Tech. Methods, **27,** 23–61.

Smith, F. 1946. Analogs of ascorbic acid. Advances in carbohydrate chemistry. Academic Press Inc., New York, **2,** 85.

STAINING METHODS FOR STUDYING INGREDIENT DISTRIBUTION IN BAKED CAKES[1]

MARTHA E. JOOSTE[2] and ANDREA OVERMAN MACKEY, *Dept. of Home Economics, Agricultural Experiment Station, Oregon State College, Corvallis, Ore.*

Received for publication June 2, 1951

ABSTRACT.—**A method is described for preparing cake crumb for sectioning and staining. Previous to embedding, the fat was stained and fixed by exposing small blocks of cake to the fumes from a 5%, freshly-prepared, aqueous solution of osmic acid (OsO_4). This was followed by dehydration in ethyl alcohol and tertiary butyl alcohol, removal of air under vacuum and infiltration with paraffin.**

Sections were cut 20 and 30μ thick and mounted with water. Wax was removed by immersion in xylene. The sections were rehydrated in a series of ethyl alcohol dilutions, from concentrated to dilute, then transferred to distilled water.

Protein was then stained pink by immersion of the slides in an acidified 0.04% water solution of eosin Y, or starch was stained blue with a dilute aqueous solution of iodine. Ten grams iodine and 10 g. KI were dissolved in 25 ml. distilled water. This stock solution was diluted for use one to two hundred times.

The relationship between protein and starch was demonstrated by staining the sections with eosin, differentiating in 50% alcohol and staining with iodine.

When slides of cake crumb were prepared in this way, the fat was stained black, the protein bright pink and the starch granules a dark blue.

When materials are to be embedded for microscopic studies, the presence of large amounts of fat creates a problem of solubility. Dehydration and clearing of tissues involve the use of fat solvents, xylene and absolute alcohol. Before these reagents can be used with bakery products such as cakes made with fat, it is essential to fix the fat in the crumb.

Carbowax embedding is unsuitable because of inadequate infiltration of the wax in the presence of large amounts of neutral fats (Firminger, 1950).

Lowe and Nelson (1939) suggested that osmic acid could be used

[1]Published as Technical Paper No. 692, with the approval of the Director of the Oregon Agricultural Experiment Station. Contribution of the Department of Home Economics.

[2]Permanent address: Department of Home Economics, University of Stellenbosch, Union of South Africa.

for fixing and staining fat in cake, and they briefly described a method for its use.

Osmic acid, being a good oxidizing agent, is readily reduced to osmium dioxide and metallic osmium. The fat in cake crumb is oxidized at the double bonds and the osmium, a heavy metal, is adsorbed by the fat. This adsorbed metal, as well as staining the fat black, protects it from being dissolved by fat solvents (Romeis, 1948).

In addition to the problem of fat-fixing and staining, alcoholic dehydration causes some hardening of tissues, and clearing with xylene is known to render specimens refractory to sectioning (Pearlman and Cole, 1951). The tendency toward crumbling of the dehydrated cake crumb was overcome by omitting xylene and clearing, instead, with tertiary butyl alcohol.

After sections have been deparaffinized, they can be stained with eosin and iodine in order to demonstrate protein and starch distribution.

The purpose of this paper is to list the steps by means of which satisfactory sections of cake crumb may be prepared for microscopic study of ingredient distribution.

Staining the Fat and Dehydrating Cake Crumb

Small blocks of cake approximately ½ × ½ × 1 cm. were placed on glass wool and arranged around a 5 ml. beaker containing 1½ ml. of a 5% freshly prepared, aqueous solution of osmic acid (OsO_4)[3]. The whole was covered with a small bell jar, 4½ inches in diameter and 2 inches in height, and the edge well sealed with stopcock grease. This was kept in the dark for seven hours, after which time the osmic acid had penetrated practically to the center of each block of cake, as shown by the black stain.

Dehydration and infiltration with paraffin were carried out as follows:

1. The blocks were placed in absolute alcohol with three changes during nine hours.
2. The air was then evacuated by placing the covered vials in a vacuum oven for two hours at 42°C. under 15 inches of vacuum.
3. The blocks were transferred to a solution containing equal parts of absolute ethyl and tertiary butyl[4] alcohol, and left for four hours.
4. The blocks were transferred to tertiary butyl alcohol which was changed three times, twice after four hours and once after 24 hours.

---

[3]Osmic acid is highly corrosive, and care must be exercised in its preparation and use. Refer to Johansen (1940).

[4]Tertiary butyl alcohol solidifies when the temperature drops below 25°C. It may be remelted by placing the container in warm water.

5. Paraffin[5] chips were added to the last change of alcohol and the vials placed in an oven maintained at 60°C. until the paraffin melted.
6. The blocks were transferred to pure melted paraffin and the paraffin replaced three or four times, every twelve hours. The vials were kept at 60°C.
7. After the last infiltration, the contents of the vials were poured into suitable glycerine-lined molds, then submerged in ice water to ensure even-textured paraffin through rapid chilling.

Cake blocks prepared in this way were trimmed down and sectioned without difficulty on a rotary microtome.

The sections were cut 20 and 30μ thick. When the protein was to be stained, the ribbons were mounted on slides with water and no fixative was used. When protein was not to be stained, Mayer's egg albumin fixative was used (Johansen, 1940). The slides were then dried on an electrically heated slide warmer for one hour, then at room temperature for at least 36 hours. To remove the wax the slides were immersed in xylene for at least 5 minutes. When slides were not to be stained a drop of thin balsam was placed on the section at this point and a cover glass mounted. The slides were placed in an incubator at 60°C. for the balsam to dry.

Staining the Protein

A dilute solution of aqueous eosin was found to stain protein a bright pink; a 0.04% solution proved to be satisfactory for this purpose. A drop of glacial acetic acid added to 100 ml. of stain helped to give a better adsorption of the dye by the protein (Cole, 1941). After the wax had been dissolved by the xylene, the slides were passed through a series of ethyl alcohol dilutions of 95, 70, 50, and 30%. The slides were kept for 5 minutes in each alcohol solution. From the 30% alcohol they were transferred to distilled water, then stained with eosin for one hour. Alcoholic dehydration was then carried out by passing the slides through the different alcohol solutions, starting with the most dilute and ending with absolute alcohol. They were then placed in xylene 100 parts, plus absolute alcohol, one part, and finally transferred to undiluted xylene. Dehydration was accomplished in approximately seven minutes. During this process, uncombined eosin was removed. Excess xylene was wiped from the slide, care being taken to avoid touching the sections. A drop of thin balsam was placed on the section and a cover glass mounted. The slides were left to dry for a few days when they were ready for microscopical examination.

[5]Fisher Tissuemat Embedding wax, m. p. 50–52°C.

Staining the Starch

An aqueous solution of iodine was made by dissolving 10 g. I_2 and 10 g. KI in 25 ml. distilled water. The stock solution when diluted one to two hundred times, stained the starch granules a distinct blue.

The procedure described for staining with eosin was followed. From the distilled water the slides were immersed in dilute iodine solution for two minutes and rinsed in distilled water. Sections stained with iodine could not be submitted to dehydration, since alcohol removes the blue color. Cover glasses were mounted in water. Stained sections may be preserved for a few days by sealing the edges with melted vaseline. The relationship between starch and protein was demonstrated to some extent by staining the sections with eosin, differentiating in 50% alcohol and staining with iodine.

Microscopic Appearance

When slides of cake crumb were prepared in this way, the fat was seen to be black, the protein bright pink and the starch granules a dark blue. Certain fats were found to gather in globules along the edge of the air spaces, while others tended to impregnate the cake crumb as well as to line the crumb at the air-cake interface. A network of pink-stained protein formed the structure of the cake crumb. The fibrous nature of the framework suggested that flour proteins were for the most part responsible for the structure. A few spherical shaped patches which stained a deep pink appeared in all sections. Since dried milk was used in the cake formula, the deep pink spots may indicate the presence of milk protein which had not been reconstituted. Blue starch granules were heavily packed in the protein structure and some appeared in the air/crumb margin partly covered with fat.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Professor Elmer Hansen, Department of Horticulture, Oregon State College, for helpful suggestions and the use of equipment.

REFERENCES

Cole, E. C. 1941. Comparative Histology. The Blakiston Company, Philadelphia. See p. 366.

Firminger, H. I. 1950. Carbowax embedding for obtaining thin tissue sections and study of intracellular lipids. Stain Techn., **25**, 121–3.

Johansen, D. A. 1940. Plant Microtechnique. New York, McGraw Hill. See pp. 7, 21 and 37.

Lowe, B., and Nelson, P. M. 1939. The physical and chemical characteristics of lards and other fats in relation to their culinary value. II. Use in plain cake. Iowa State College Research Bulletin 255, p. 164.

Pearlman, R. C., and Cole, Buell C. 1951. Softening of hard tissue for sectioning. Stain Techn., **26**, 115–18.

Romeis, B. 1948. Mikroskopische Technik. 15th Ed. Leibniz Verlag, Munich. See p. 248.

PAPER CHROMATOGRAPHY FOR ANALYSIS OF A DYE MIXTURE

ARTHUR J. EMERY, JR. and ELMER STOTZ, *Laboratories of the Biological Stain Commission, Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York*

Received for publication June 19, 1951

ABSTRACT.—**A paper chromatographic method for the identification and separation of stain components is proposed. Using an alkaline butanol-water solvent and the descending technic, a commercial product, known as "Triosin", believed to consist of three (undetermined) dyes, has been separated into five components. Three of the components were apparently parts of a typical eosin Y commercial sample, and the other two were orange G and erythrosin B. By means of elution of the components from the paper, followed by spectrophotometric analysis, it was possible to reconstitute a mixture which appeared to be identical with Triosin in spectrophotometric, chromatographic, and histological properties.**

It is a well known fact that biological stains are mixtures which are difficult to resolve into pure components. To date, methods such as recrystallization and freezing out have been used, but these methods do not assure complete purification. In recent years there has been increased interest in the use of ion-exchange resins, filter paper chromatographic columns and alumina columns for purification and separation of chemicals, although little has been published about these methods as applied to dyes. (Ruggli and Jensen, 1935, 1936.) As a matter of historical interest, it is well to mention the work of Schönbein (1861) which was probably the first use of paper chromatography for separation of dyes. Oddly enough, practically nothing was done to develop the method as pertaining to dye separation until very recently – by Rao, Shah, and Venkataraman (1950).

Most of the methods listed above are not very satisfactory, for they are too time-consuming, involve somewhat difficult technics and require too large a sample. A more desirable method would be one that requires only a minimum of equipment, relatively short time period, consists of a simple technic, produces good separation of components, and requires only small amounts of sample. The relatively recent modifications of paper chromatography seem to offer good possibilities.

In view of the fact that the literature contains very little about the use of paper chromatography for separation, identification and quantitative analysis of dyes, the authors undertook preliminary

studies to determine the possibilities of this technic as a useful tool for further research by dye chemists and analysts.

A product of practical value but known to be a mixture of undetermined dyes was selected for this study. This product, called "Triosin" (Galigher, 1934), was believed to consist of at least three dyes, thus giving an ideal sample to test the proposed technic. The object of the study was to separate the mixture of dyes into its pure dye fractions, to analyze the dye fractions and to determine the quantitative amounts of each, as an illustration of the applicability of paper chromatography to dye mixtures.

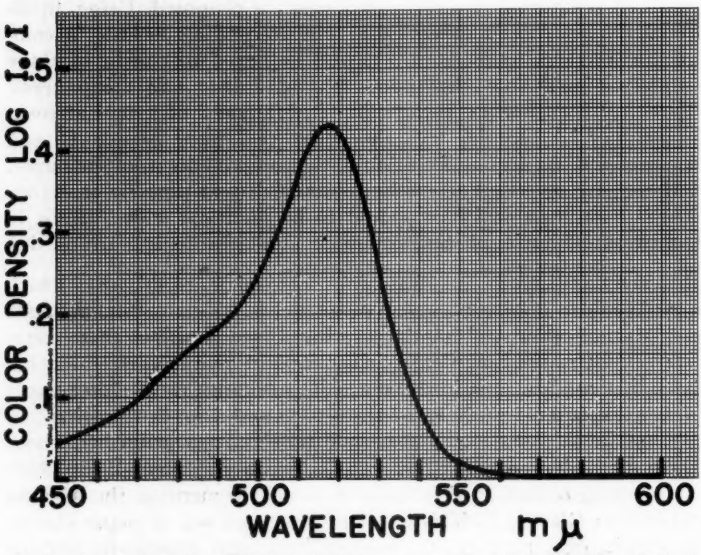


FIG. 1. — Spectral curve of Triosin (0.005 mg. per ml. 0.01% Na_2CO_3 solution).

PROCEDURE

Spectrophotometric analysis of Triosin was made as follows: A stock solution was made by dissolving 50 mg. Triosin in 100 ml. of distilled water by shaking on a Boerner shaker (Arthur H. Thomas Co., Philadelphia, Pa.), then diluting to 250 ml. with distilled water. To 5 ml. of this stock solution were added 2 ml. of 1% Na_2CO_3 and the solution diluted to 200 ml. with distilled water. Approximately 3 ml. of this solution were then placed in a Corex cuvette (1 cm. light path) and analyzed spectrophotometrically (Beckman DU Spectrophotometer) from 450 to 600 mμ. The solvent cell consisted of approximately 3 ml. of a Na_2CO_3 solution made by diluting 2 ml. of 1% Na_2CO_3 to 200 ml. with distilled water. Fig. 1 shows the spectral curve obtained by this analysis.

Chromatographic separation of Triosin fractions was carried out by means of an alkaline butanol-water system using Whatman No. 1 filter paper, with the descending technic, at room temperature (25°C.). The alkaline butanol-water mixture was made as follows:

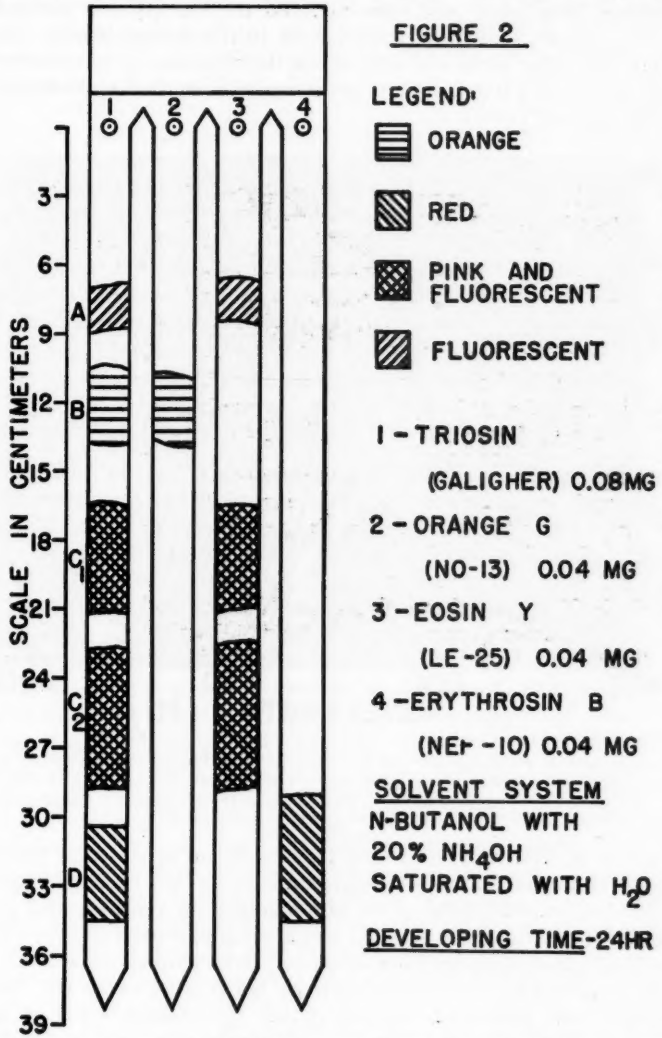


FIG. 2. — Chromatographs of Triosin, orange G, eosin Y, and erythrosin B. Fractions from top to bottom of Triosin chromatograph are here designated as A, B, C_1, C_2, and D.

80 ml. *n*-butanol plus 20 ml. NH_4OH (Sp. gr. 0.90) plus 100 ml. distilled water were shaken together and allowed to settle. The bottom water layer (saturated with alkaline butanol) was placed in a beaker at the bottom of the chamber to keep the atmosphere saturated with water and ammonia, and the top alkaline butanol layer (saturated with water) was placed in the solvent trough into which the paper strip was dipped for development. The chamber was sealed with a starch-glycerol paste suggested by Burton, Zaffaroni and Keutmann (1951).

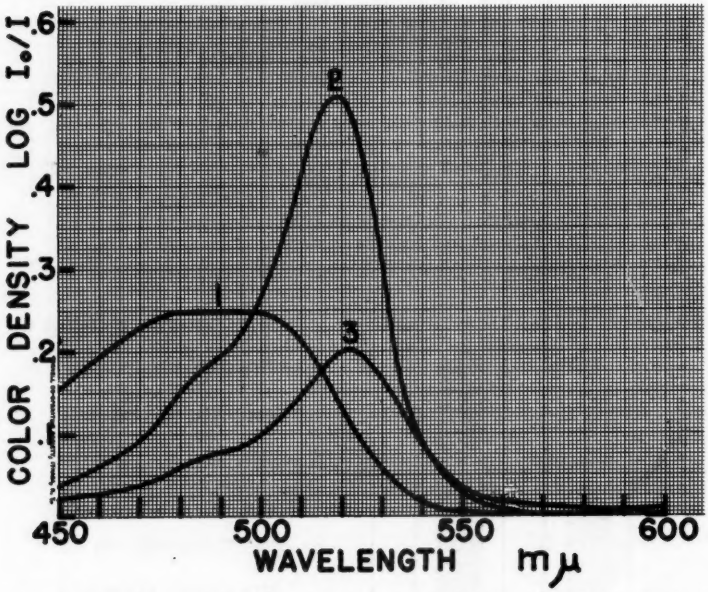


FIG. 3. — Spectral curves of Triosin fractions. Curve 1 is that of fraction B dissolved in 5 ml. of water; Curve 2 is a mixture of fractions A, C_1, and C_2, dissolved in 15 ml. of water; Curve 3 is from fraction D dissolved in 5 ml. of water.

Sheets of Whatman No. 1 filter paper were cut as shown in Fig. 2. The overall dimensions were approximately 45 by 7 cm., the length depending on the height of the chromatographic chamber and the width depending on the number of the samples desired to test. The sheet was recut into separate strips for each sample; the width of each strip was 1.5 cm. and the distance between strips 0.5 cm. The samples were placed on the filter paper approximately 6.5 to 7.0 cm. from the end of the paper to be inserted into the developing solvent phase. Dye solutions to be tested were made up in concentrations of 2 to 4 mg. dye per ml. of distilled water. Small samples of

the dye (0.04 to 0.08 mg.) were placed on the separate strips, equidistant from the upper end of the filter paper, by means of a graduated capillary pipette and dried quickly by a capillary stream of filtered air. This procedure confines the dye to a smaller area, thus producing better separation and less diffusion or "tailing". The top of the filter paper was then placed in the developing solvent phase in the trough at the top of the chromatographic chamber and the strips allowed to hang freely in the chamber from a distance of at least 1 cm. above the "spotted" samples. As the solvent reached the bottom of the strips, the excess dropped to the bottom of the chamber. Individual dye fractions were collected by appropriate changes of beakers placed underneath the strips. This method was made quantitative by drying the fractions at room temperature, in a desiccator or by an air stream, then diluting to the concentrations desired for proper spectrophotometric analysis. Sufficient dilution for accurate spectrophotometric analysis was secured with 5 to 15 ml. of distilled water, depending on the dye and the amount "spotted" on the chromatograph. Results of spectral analysis of fractions from Triosin are shown in Fig. 3.

Results

Examination of the spectral curve of Triosin (Fig. 1) did not enable the authors to estimate the number of dyes composing the mixture. The curve is fairly characteristic of eosin Y which produces an absorption peak at 518–519 mμ (in the solvents used as mentioned under Procedure) with a slight shoulder at approximately 470–495 mμ. Thus there was no obvious indication that it was a mixture of dyes.

Chromatographic studies, however, made it clear that Triosin was a mixture. As seen in Fig. 2 there were five distinct fractions which will be referred to in the order of increasing rate of movement: A – fluorescent and nearly colorless; B – orange; C_1 and C_2 – pink and fluorescent; and D – red. Each of the five fractions was collected, dried and dissolved in distilled water, and analyzed spectrophotometrically. The orange fraction (B) and the red fraction (D) produced spectral curves that were characteristic of orange G and erythrosin B respectively, (Fig. 3, Curves 1 and 3). Of the two pink, fluorescent fractions (C_1 and C_2) and the fluorescent fraction (A), fraction C_2 resembled eosin Y in the position of its peak, but the shape of the curve was not characteristic of eosin Y. By combining all the fluorescent fractions (A, C_1 and C_2), a spectral curve characteristic of eosin Y was obtained (Fig. 3, Curve 2). It was then confirmed by chromatography (Fig. 2) that eosin Y (LE–25) consists of three pink fluorescent fractions. Fig. 2 also shows the results of orange G (NO–13), eosin Y (LE–25), and erythrosin B (NEr–10) after being chromatographed along with Triosin for comparative analysis. As can be seen, fractions in the Triosin mixture moved to distances correspond-

ing to similar distances for the "pure" dyes believed to compose the mixture. It seems clear therefore that Triosin consists of the three dyes: orange G, eosin Y, and erythrosin B.

With identification of the fractions assured (spectrophotometrically and chromatographically) quantitative determinations of the fractions were made. Chromatographic separation of a known amount of Triosin (0.04–0.08 mg.) was carried out and fraction D was collected in one beaker, fractions A, C_1 and C_2 in a second, and B in a third. After evaporating to dryness and proper dilution, spectral analyses were carried out to determine the amount of "pure" dye in each fraction. This was determined by comparing the density readings at the peak with the density readings at the respective peak for pure dye solutions of known concentration. The amounts of orange G, eosin Y and erythrosin B were calculated. From research data of the Biological Stain Commission, it was found that for 0.005 mg. of each of the "pure" dyes per ml. of water, density readings at the peak were obtained as follows:

		Peak (mμ)
Orange G	— 0.240 —	492–497
Eosin Y	— 0.680 —	518–519
Erythrosin B	— 0.562 —	522–523

The absorption values at the peak and data for calculation of mg. of pure dye per ml. of water solution for the three dye fractions were as follows:[1]

Dye Fraction	Dye	Dilution with water (ml.)	Density at peak	Density on equal dilution basis (5 ml.)	mg. pure dye per ml. of water solution
B	Orange G	5	0.249	0.249	5.19×10^{-3}
A, C_1 and C_2	Eosin Y	15	0.508	1.524	11.22×10^{-3}
D	Erythrosin B	5	0.201	0.201	1.79×10^{-3}

This indicates that the ratio of "pure" dyes by weight is 2.89:6.28:1 for orange G, eosin Y and erythrosin B, respectively. An experimental combination of 28% orange G (NO–13), 62% eosin Y (LE–25) and 10% erythrosin B (NEr–10) calculated as pure dye was made by thoroughly mixing the above dyes, thus producing a mixture which closely approximated the ratio of 2.89 : 6.28 : 1 as found experimentally. Fig. 1 and 4 show the comparison of the spectral analysis of equal weights of Triosin and the experimental mixture. The results show that it is very nearly identical with Triosin by spectrophotometric analysis.

Comparative chromatography of the experimental mixture and Triosin produced virtually identical results both in the distance moved by the respective fractions and in the apparent quantity of each[2].

[1]Calculated by multiplying the actual density by 100/% purity.

[2]It should be mentioned that when the experimental mixture was made, it was found that the commercial samples of eosin Y varied in their chemical composition

The final test was to compare the experimental mixture and Triosin in their staining properties. Comparison studies were made by various staining methods[3]. Paraffin sections with iron hematoxylin and (a) Galigher's Triosin and (b) experimental mixture, were tested at pH 5.5 according to Galigher's directions, (Galigher, 1934, p. 209). Differentiation was about equal in the two samples and the two samples were almost interchangeable at given staining times. Tests on SUSA-fixed monkey intestine were also made and the two mixtures behaved as though the ingredients were the same and in the same balance.

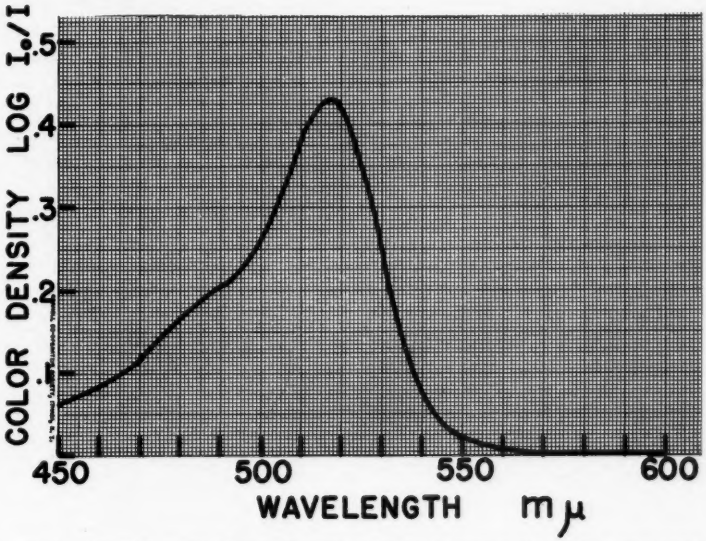


FIG. 4. — Spectral curve of experimental mixture of orange G, eosin Y and erythrosin B (0.005 mg. of mixture per ml. 0.01% Na_2CO_3 solution).

DISCUSSION

The data presented above seem to indicate that Triosin consists of approximately 28% orange G, 62% eosin Y and 10% erythrosin B in terms of actual dye content. After comparative tests, spectrophotometrically, chromatographically, and histologically, it appears to be quite conclusive that the analysis is correct.

---

with the different manufacturers. By chromatographic studies it was found that the eosin Y of National Aniline Co. contained an additional fraction not present in most samples from other companies or in Galigher's Triosin, therefore eosin Y made by Hartman-Leddon Co. was used to make the experimental mixture.

[3]The authors wish to thank Dr. John L. Mohr (Associate Professor of Zoology, University of Southern California) for his cooperation in the histological aspects of this work.

This study indicates the value of paper chromatography as a technic for separation, identification and semi-quantitative analysis of dyes and dye homologs. The method may prove very useful to the dye chemists and analysts in solving many of the heretofore very difficult or impossible purifications, separations and identifications. However, it is understandable that the solvent systems and other steps of the technic will have to be modified according to the dyes and conditions under which they are to be studied.

Conclusions

1. A descending paper-chromatographic technic, using a solvent system of equal parts of alkaline *n*-butanol and distilled water, has been used successfully on an unknown mixture sold commercially under the name of "Triosin", which was selected as a test material for analysis.

2. By this method the unknown was separated into five fractions.

3. Spectrophotometric analyses show the five to be fractions of three dyes which are orange G, eosin Y and erythrosin B.

4. Quantitative analyses of chromatographic fractions indicated that the test product is a mixture of approximately 28% orange G, 62% eosin Y and 10% erythrosin B.

5. An experimental mixture of the three dyes in the amounts shown above appeared to give identical results with that of Triosin when compared spectrophotometrically, chromatographically and histologically.

6. Paper chromatography is a technic which can successfully be applied to dye research for separation of dyes and dye homologs, identification and semiquantitative analyses of the fractions.

REFERENCES

Burton, R. B., Zaffaroni, A., and Keutmann, E. H. 1951. Paper chromatography of steroids II. Corticosteroids and related compounds. J. Biol. Chem., **188**, 763–71.

Galigher, A. E. 1934. The Essentials of Practical Microtechnique in Animal Biology. Albert E. Galigher, Inc., Berkeley, Calif.

Rao, N. R., Shah, K. H., and Venkataraman, K. 1950. Chromatographic separation of dyes. Current Science (India), **19**, 149–50.

Ruggli, P., and Jensen, P. 1935. Die chromatographische Adsorptionsanalyze in Anwendung auf wässrige Lösungen kunstlicher organischer Farbstoffe. Helvetica Chimica Acta, **18**, 624–43.

Ruggli, P., and Jensen, P. 1936. Chromatographische Adsorption von Teerfarbstoffen und Zwischenprodukten. II. Derivate des α- und β-Naphthols, Helvetica Chimica Acta, **19**, 64–8.

Schönbein, Ch. F. 1861. Über einige durch die Haarröhrchenanziehung des Papieres hervorgebrachten Trennungswirkungen. Verh. d. Naturf. Ges. i. Basel, Teil III, 249.

RAPID PREPARATION OF THICK SECTIONS OF FLESHY PLANT TISSUES

R. M. Reeve, *Western Regional Research Laboratory,*[1] *Albany, California*

Received for publication June 30, 1951

Abstract.—**Methods are described for permanent micro-slide preparations of soft, large-celled plant tissues such as ripe fruit. Thick sections (200–800 μ) cut on a sliding microtome are aspirated in an aqueous killing agent; after fixing and washing, the sections are dehydrated and cleared in an alcohol-xylene series. Infiltration with 20, 30, and 40% solutions of mountant prior to mounting the sections is necessary to avoid too abrupt changes in the cleared tissues. Several staining methods have been successfully used for different purposes. The final preparations showed nearly perfect preservation of intact cells and intercellular spaces in their 3-dimensional structure.**

The large cells and intercellular spaces of many soft plant tissues such as found in pithy stems, tubers, and physiologically senescent, ripe fruits, present difficult problems in histological technic. Thin sections prepared by the usual methods from paraffin-embedded samples do not reveal the three-dimensional structure of the tissue, and the large, vacuolate cells are more or less distorted as a result of compression by the microtome knife. Many of the storage parenchyma cells of these tissues are 100 μ or more in diameter when mature, so that thicker sections (200 to 800 μ) than obtainable from paraffin-embedded samples are necessary to show structural organization. Collodion technics have been recommended for these purposes (Johansen, 1940) but they require time-consuming operations. Rapid, direct and more adaptable methods are described here.

General Method

In investigations on apples and other fruits, it was found that good permanent preparations of thick sections could be obtained by employing technics similar to those described by Foster (1949) and Morley (1949) for intact leaves. A general technic schedule was devised to suit various conditions. The main features of this schedule, as used for fresh sections of potato tubers and ripening fruits, are as follows:

1. Fresh sections were cut on a sliding microtome at an optimum

[1]Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

thickness ranging from 200 to 800 μ, depending on size of cells, intercellular spaces and plane of sections; they were placed immediately in an aqueous killing agent of the CRAF type and then mildly aspirated in the reagent for about 10 minutes or until they were translucent.

2. After aspiration the sections were left in the killing agent for an appropriate time to complete fixation. They were then washed in slowly running water for 30 minutes or longer in a wide-mouthed bottle capped with gauze.
3. The washed sections were stained in an aqueous mixture of gentian violet and safranin O (ca. 0.1–0.2% each) for 30 to 60 minutes at room temperature, or for 10 to 12 minutes at 37.5°C. This provided a good general tissue stain which was suitable for structural characteristics. However, the time required for adequate or optimum staining with different tissues varied greatly and could be determined only by trial. Other staining methods discussed later were used also.
4. Excess stain was washed out by passing the sections through several changes of water, although some dye continued to leach out during alcohol dehydration of the sections.
5. Senescent fruit tissues required cautious handling in the dehydration stages. The most satisfactory series of ethyl alcohol concentrations used was 10, 20, 30, 50, 75, 85, 95 and 100%. The sections were left in each solution about 5 to 7 minutes. At least 3 additional changes were made in clean absolute alcohol.
6. Clearing also was found to be a critical stage with fruit tissues. Ratios of alcohol to xylene used successfully were: 3:1, 2:1, 1:1, 1:2, and 1:3, followed by at least 3 changes in pure xylene. The sections were left at least 3 or 4 minutes in each solution and kept submerged, except for transfer, to guard against introduction of air into the intercellular spaces.
7. Step-wise infiltration of the sections with balsam or similar mountants was found necessary to avoid distortions caused by direct change from xylene to the conventional 60% solution of the mounting medium. Twenty, 30, 40, and 60% solutions of the mountant in xylene were used for this purpose and the sections were left submerged at least 10 minutes in each solution. If, at this stage, the sections showed any opacity, crinkling, or appreciable shrinkage, they were discarded and the next series of sections was treated with greater care in the prior stages to insure complete removal of water and replacement of alcohol with xylene.
8. Mounting the sections on microslides also required careful handling to avoid introduction of air. Difficulties were minimized by covering an area of the slide with a 40% solution of the mountant to which the section was transferred and im-

mediately covered with a few drops of a thickened mountant (ca. 80%). Then, by warming the cover glass in a clean alcohol flame just before placing it over the section, a uniform flow of the mountant along the lower surface of the cover glass could be obtained.

In using this schedule for small sections of ¾ inch or less in width, the solutions were kept in Syracuse watch glasses and the individual sections were transferred with a section lifter. When large numbers of sections or when entire transverse or longitudinal sections of apples were processed, the solutions were prepared in petri dishes and the sections were sometimes transferred by means of shallow screen baskets.

Alternate Technics

"Firming" with a calcium salt: Some ripe fruits are too soft to be successfully microtomed in the fresh condition. In addition, well ripened apples may be sufficiently mealy that even when fresh sections can be obtained they are difficult to handle. The middle lamellae of such tissues are structurally weak and the senescent condition of their cell contents may cause protoplasmic shrinkage when the sections are processed further. It was found that these difficulties could be minimized to some extent by the use of a calcium salt to firm the tissue.

Blocks of soft fruit tissues were soaked for periods up to an hour in either 0.5 or 1% aqueous calcium chloride solution prior to sectioning. In other instances the tissue blocks were mildly aspirated for about 20 minutes in the calcium chloride solution. Usually the rigidity of the tissue was sufficiently increased by these treatments that fresh sections could be readily cut on the sliding microtome, i.e., the formation of calcium pectates resulted in a "firming" of the middle lamellae. In some cases the pectic changes which accompanied ripening had progressed to such an extent that insufficient tissue firming could be obtained. The only methods by which thick sections of these over-ripe tissues could be obtained was with the more tedious and time-consuming collodion technics (Johnson, 1940).

Apples which showed a tendency toward mealiness, but which could be sectioned in the fresh condition, were firmed after sectioning. This was accomplished by killing and fixing the sections in an 8–10% dilution of commercial formalin containing about 0.5% calcium chloride. After mild aspiration in such a reagent the sections were appreciably firmed and were much easier to handle individually in the staining and dehydration procedures. It was noted that in the calcium-treated sections there was less tendency for protoplasmic shrinkage to develop in the highly vacuolate parenchyma cells. It seems likely, then, that the protoplasmic gels also were appreciably firmed by the calcium salt.

These calcium treatments did not adversely influence the staining quality of the sections and in some cases the staining of finer protoplasmic strands appeared to be enhanced.

Combined fixing and staining: The chemical fixing and localization of some cellular components can be accomplished with special killing agents and without subsequent use of histological dyes. For instance, a 10% aqueous solution of formalin containing about 2 or 3% ferrous sulfate provides a killing agent which fixes tissue tannins (Johansen, 1940; Zirkle, 1932). Although this reagent does not provide a selective chemical test for tannins or other phenolic compounds (Reeve, 1951) it was found to provide good cytological

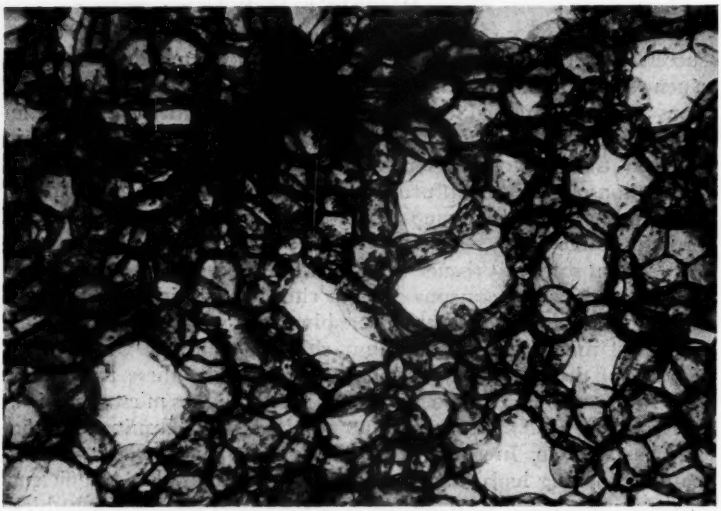


FIG. 1. — Tangential section ca. 300 μ thick of flesh from Newtown Pippin apple stained with gentian violet and safranin O. × 40.

localization where certain phenolic compounds were sufficiently concentrated to yield easily observed precipitates with the iron salt.

In thick sections of apple tissues killed with the above reagent, and prepared without other staining, the iron-phenolic precipitates were not pronounced except in the vascular tissues where the much smaller cells accentuated the "staining" effect. In these cells (phloem and phloem parenchyma) the precipitates appeared as dark, granular deposits in the protoplasm as well as in the vacuoles. In the larger storage parenchyma cells the precipitates appeared as a finer flocculation in some vacuoles. All cellulose walls and the middle lamellae were stained gray by this reagent.

Ferric chloride frequently is recommended as a reagent for cellular localization of tannins (Johansen, 1940). Although a characteristic blue-green iron-phenolic precipitate results in test tube reactions, the use of ferric chloride for histochemical detection of tannins does not always provide satisfactory results (Reeve, 1951). For instance, when ferric chloride, instead of ferrous sulfate, was used in the formalin fixation of fresh sections of apple tissues as described above, it was found that the vacuoles of many parenchyma cells retained the natural yellow color of the iron salt, even after the sections had been mounted in balsam, and seldom showed any iron-tannin precipitates. To some extent this clear yellow in the contents of intact cells en-

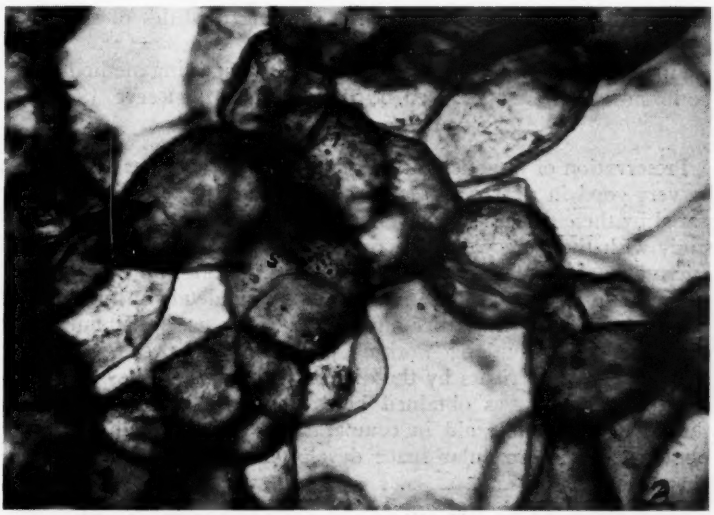


FIG. 2. — Radial section ca. 300 μ thick of flesh from Gravenstein apple. Fixed in ferrous sulfate and formalin. Stains: Aniline blue, gentian violet and safranin O. S-starch granules. × 150.

hanced the appearance of the sections when they were counterstained with safranin and other dyes, but the use of ferric chloride appeared to have no other advantages.

Other staining technics: The general tissue staining with safranin and gentian violet, as described in the schedule was not always found satisfactory. For instance, the quality of staining which presented a good appearance for observations wth a stereoscopic microscope was not always suitable for study with a compound miscroscope. Some problems also were encountered in staining cell contents adequately without overstaining the cell walls. Since the possible causes of these problems differ considerably according to age of the tissue,

no specific staining method is recommended as superior to another.

Other technics which were used for general tissue staining and found to give good results were: (1) Delafields' hemotoxylin (½ strength in tap water) for 5–10 minutes followed with either an aqueous or alcoholic (60%) solution of 0.1% safranin as a counterstain at the proper place in the schedule; (2) aqueous or alcoholic safranin followed by a fast green solution in the 1:3 alcohol-xylene stage of the schedule: and (3) tannic acid (0.1%) in either aqueous or alcoholic solution for 2–3 minutes followed, after thorough rinsing of the sections, by a similar solution of ferric chloride. Safranin was used as a counterstain. The tannic acid and ferric chloride treatment as it was used here for apple parenchyma, also provided a mordant to improve the staining qualities of some of the cell contents.

Further details or suggestions for alternative staining methods can be found elsewhere (Johansen, 1940; Morley, 1949; Reeve, 1948).

Conclusions

Preservation of intact cells and intercellular spaces was judged to be very good in nearly all sections of fruit and potato tissues prepared by these direct methods. The thick sections are well suited to study with the stereoscopic microscope. The use of the compound microscope for such thick sections has practical limitations, but where necessary some of the finer protoplasmic detail in intact cells can be examined with either high dry or low power oil immersion objectives of fairly long working distance. The preservation of structural characteristics by the direct killing and fixing of freshly cut thick sections, as obtained here even with senescent tissues, should be a valuable aid in comparative studies of cellular morphology and intercellular space development in maturing parenchyma tissues.

In addition to the advantages of good structural preservation these methods might be profitably exploited for histochemical investigations. For instance, the effects of pH of different killing agents on the cellular contents have been widely investigated for plant meristems (Zirkle, 1932) but little attention has been given to histological fixation in mature parenchyma. Consequently, little is known cytologically about senescent and metabolically active stages of fruit parenchyma. Also, selective chemical reagents which provide a colored or dark, insoluble precipitate with certain of the cell contents should be more readily adaptable to the methods described here than to the conventional paraffin and collodion technics of plant histology.

References

Foster, A. S. 1949. Practical Plant Anatomy. D. VanNostrand Co., New York.

Johansen, D. A. 1940. Plant Microtechnique. The McGraw Hill Book Co., Inc. New York.

MORLEY, THOMAS. 1949. Staining of plant materials cleared in NaOH. Stain Techn., **24**, 231–5.

REEVE, R. M. 1948. An adaptable staining schedule for plant tissues. Stain Techn., **23**, 13–15.

———. 1951. Histochemical tests for polyphenols in plant tissues. Stain Techn., **26**, 91–6.

ZIRKLE, C. 1932. Vacuoles in primary meristems. Zts. Zellforsch. Mikres. Anat., **16**, 26–46.

ETHYLENIC REACTION OF CEROID WITH PERFORMIC ACID AND SCHIFF REAGENT[1]

R. D. LILLIE, *National Institutes of Health, Bethesda 14, Md.*

Received for publication July 5, 1951

ABSTRACT.—**On oxidation with performic (or peracetic) acid ceroid becomes Schiff positive. This reaction is prevented by prior bromination, but not by acetylation. Interposition of bisulfite, semicarbazide or phenylhydrazine blockades after the oxidation and before the leucofuchsin prevents the positive Schiff reaction on short exposure, but a long (3 hour) Schiff bath overcomes the blockade in all three instances. Prior acetylation blocks the periodic acid Schiff reaction of ceroid, but bromination does not.**

These findings indicate the presence in ceroid of ethylenic groupings as well as 1,2 glycols.

INTRODUCTION

The yellow to brown, coarsely granular pigment, ceroid, of the choline-deficiency hepatic cirrhosis of rats which we have described (Lillie, et al., 1941, 1942, 1944, 1948) is basophilic, Sudanophilic in paraffin sections, acid-fast, isotropic, iron negative, and highly resistant to solution in water, alcohol, fat solvents, and dilute aqueous acids and alkalies. It reduces osmium tetroxide and diammine silver carbonate. It retains Mallory's hemofuscin stain and Weigert's myelin stain. It does not reduce ferric ferricyanide. It displays a greenish yellow fluorescence in 3650–3660 A. ultraviolet light, which changes slowly to yellowish white when frozen sections are used, while in paraffin sections the fluorescence is golden brown. The Gmelin reaction is negative.

The similar, acid-fast pigment of butter-yellow cirrhosis in rats differs in its lack of basophilia (Edwards and White, 1941; Smith, Lillie and Stohlman, 1943). Ceroid from various conditions and species was later described as HIO_4-Schiff positive (Elftman et al. 1949; Lee, 1950; Lillie, 1951a), both with and without antecedent diastase digestion, sometimes directly Schiff positive without oxidation (after fixation in Stieve's acetic sublimate formalin, Lee, 1950) and often Bauer and Casella positive (Lillie, 1951a). Lee found basophilia variable, even in the same animal.

The lipoprotein theory of the nature of ceroid advanced by Ed-

[1]From the Histochemistry Unit, Section on Pathologic Anatomy, Laboratory of Pathology and Pharmacology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, Public Health Service, Federal Security Agency.

wards and White (1941) and György and Goldblatt (1942) seems less tenable than that of the partially oxidized and polymerized fat suggested by us (Lillie et al, 1944) and indirectly supported by the behavior of cod liver oil and linseed oil under chromate oxidation (Endicott, 1944) and by the nonappearance of ceroid when choline-deficiency cirrhosis is induced with diets lacking unsaturated fats (Endicott, Daft and Sebrell, 1944). Elftman's (1949) negative histochemical tests for protein also tend to support this view.

Recently, on encountering the statement in Hickinbottom's text (1941) that aliphatic peracids were used in the synthesis of glycols from olefines, it seemed that this might offer a feasible histochemical procedure for the demonstration of unsaturated compounds, and thereby permit the testing of ceroid for the presence of C=C groupings. For this purpose the procedures of Greenspan (1946) for the production of performic and peracetic acids with 30% hydrogen peroxide were adopted. This method of production of glycols from olefines rests on the works of Scanlan and Swern (1940), Swern *et al.* (1945), Findley *et al.* (1945), who used both performic and peracetic acids.

Experimental

In the first trial, sections of formalin fixed, cirrhotic rat liver were used. After deparaffinizing, part of the sections were acetylated 24 hours in an acetic-anhydride:pyridine mixture (4:6) at 25°C. Then, acetylated and unacetylated sections were placed both in performic and in peracetic acids (Greenspan, 1946) for 16 hours, washed, and with acetylated and unacetylated controls, put through the usual HIO_4-Schiff procedure, using a 10-minute bath in 0.69% (.03 *M*) KIO_4 plus 0.3% HNO_3. After acetylation, ceroid failed to react in the HIO_4-Schiff controls, indicating that the acetylation technic employed had successfully blocked 1,2-glycols, but reacted moderately after both aliphatic peracids. In the unacetylated series, ceroid reacted moderately in the HIO_4-Schiff controls and strongly after the aliphatic peracids.

Since the performic acid seemed to give a more vigorous reaction, further experiments were conducted with it alone.

In the second series of experiments it was found that a 16-hour exposure to performic acid produced a positive Schiff reaction of ceroid even when the following periodic acid oxidation was omitted. With this omission, the confusing positive reactions of glycogen, collagen, reticulum and mucin disappeared. A 24-hour acetylation as above failed to prevent the performic acid Schiff reaction of ceroid, while a 1 hour bromination (1 ml. bromine in 39 ml. carbon tetrachloride) completely blocked it. The same bromination had no inhibitory effect on the HIO_4-Schiff reaction of ceroid, while the same acetylation completely prevented it.

Iodination for 6 hours in 5% iodine, 10% KI in 70% alcohol did not block either the periodic-acid- or the performic-acid-Schiff reaction.

On exploration of the necessary duration of oxidation in the performic acid reagent, a 60- to 90-minute period was found adequate to give practically optimum intensity of coloration. Shorter, 10- to 30-minute oxidations with performic acid gave more vigorous reactions when the performic acid had first aged 1½ to 2 hours than when it was freshly mixed. This agrees with Greenspan's finding that the maximum concentration of performic acid was reached only after about an hour. A 48-hour exposure, changing to fresh performic acid mixture at the end of the first 24 hours, did not destroy the Schiff reactive material.

With shorter oxidation periods, acetylation for 24 hours impaired the intensity of the following performic-acid-Schiff reaction of ceroid. Prolonged exposures to pyridine alone (48 hours at 37°, 4 hours at 58°C.) did not weaken the performic-acid-Schiff reaction. This retardation of the reaction after acetylation may possibly be due to diminished penetrability by aqueous reagents consequent on the esterification of the coincidentally present 1,2-glycol groups.

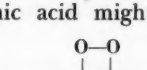
TABLE 1. SCHIFF REACTIONS OF CEROID AFTER OXIDATION WITH PERMANGANATE PERFORMIC ACID AND PERIODIC ACID.*

Acetylation	Bromination	Oxidation	Schiff reaction
.....	.....	.....	—
.....	.....	$KMnO_4$	+ + to + + +
24 hr.	.....	$KMnO_4$	± to + +
.....	1 hr.	$KMnO_4$	± to + +
24 hr.	1 hr.	$KMnO_4$	—
.....	.....	HIO_4 10′	+ +
24 hr.	.....	HIO_4 10′	—
.....	1 hr.	HIO_4 10′	+ +
.....	.....	HCO_3H 90′	+ to + +
24 hr.	.....	HCO_3H 90′	±
24 hr.	.....	HCO_3H 18 hr.	+ +
.....	1 hr.	HCO_3H 90′	—
.....	1 hr.	HCO_3H 18 hr.	—
.....	.....	HCO_3H 2×24 hr.	+ to + +

*Acetylation was done in an acetic-anhydride:pyridine mixture (4:6). Bromination was done in a 1:39 volume mixture of bromine and carbon tetrachloride. At least 3 different animals were used on each trial and multiple oxidation intervals were employed with performic acid and permanganate. Periodic acid oxidations were done in parallel with each performic acid oxidation on all of the acetylation and bromination tests. Parallel $KMnO_4$ oxidations were done for 5, 10, 20, 30 and 60 minutes, without significant variation in the intensity of the reaction. The above tabulation does not exactly represent any single experiment, but is an attempt to average the results of a number of different trials.

Since organic peracids are said to convert ethylenic groups first to epoxides, and then, by hydrolysis (perhaps through an ester stage) to 1,2-glycols, and the production of aldehyde is not commonly

noted, it was thought that the positive Schiff reaction produced by the action of performic acid might be due to a fat peroxide



such as is formed in paint oxidation (Rieche, 1931), rather than to aldehyde.

Accordingly two groups of sections were oxidized respectively for 10 minutes in 0.03 *M* periodic acid and for 90 minutes in performic acid. Then, after washing, paired sections from each oxidation group were subjected to 16-hour immersion in 0.15 *N* HCl containing 1.9% $Na_2S_2O_5$ (0.1 *M*), again washed and then reacted as usual with Schiff reagent. The strongly positive Schiff aldehyde reaction of retinal rod acromere lipoid (Lillie, 1951b) by both methods was prevented entirely by this bisulfite blockade, that of ceroid was greatly weakened. In both groups alike, the Schiff reaction after this sulfite blockade was again rendered fully positive by oxidations with 3% hydrogen peroxide or with Weigert's 1% iodine solution (Lillie, 1948, p. 69), or by a prolonged (2 hour) exposure to Schiff reagent, which will overcome sulfite blockade (Lillie, 1951a). Treatment after periodic acid or performic acid oxidation with other reducing agents (5% oxalic acid for 5 minutes, 1% hydroquinone for 5 minutes) did not interfere with the subsequent positive Schiff reaction of ceroid or of retinal rod acromere lipoid in either case.

TABLE 2. EFFECT OF SULFITE, SEMICARBAZIDE AND PHENYLHYDRAZINE BLOCKADE AFTER PERFORMIC AND PERIODIC ACID OXIDATIONS ON THE SUBSEQUENT SCHIFF REACTIONS OF CEROID (c) AND RETINAL ROD ACROMERE LIPOID (a).*

Oxidant	Blocking Agent	Unblocking Agent	Schiff Reaction 5′		Schiff Reaction 3 Hr.	
			(c)	(a)	(c)	(a)
HCO_3H	....	....	++	++	++	++
HCO_3H	Sulfite	....	±	—	++	++
HCO_3H	Sulfite	H_2O_2	++	++		
HCO_3H	Semicarbazide	....	±	—	++	++
HCO_3H	Phenylhydrazine	....	—	—	++	++
HCO_3H	Phenylhydrazine	H_2O_2	—	—		
HIO_4	....	....	++	++	++	++
HIO_4	Sulfite	....	±	—	++	++
HIO_4	Sulfite	H_2O_2	++	++		
HIO_4	Semicarbazide	....	±	—	++	++
HIO_4	Phenylhydrazine	....	—	—	++	++
HIO_4	Phenylhydrazine	H_2O_2	—	—		

*Sulfite blockade was 16 hr. in 1.9% $Na_2S_2O_5$, 0.15 N HCl. Semicarbazide blockade was 18 hr. in 5% semicarbazide hydrochloride. Phenylhydrazine blockade was 30 min. in 5% phenylhydrazine hydrochloride. H_2O_2 unblocking was 5 min. in 3% hydrogen peroxide.

Similarly a 30-minute bath in 5% aqueous phenylhydrazine hydrochloride, after oxidation with performic or periodic acid, completely

prevented the subsequent aldehyde reaction of ceroid and of rod acromere lipoid with Schiff reagent (5 minutes) in both instances. Control, unblocked preparations gave strongly positive reactions.

Interpolation of an 18-hour bath in 5% semicarbazide hydrochloride between oxidation either by periodic or by performic acid and the 5-minute bath in Schiff reagent, reduced the coloration of ceroid to a light pink and abolished the Schiff aldehyde reaction of retinal rod acromere lipoid completely. Like bisulfite blockade, both phenylhydrazine and semicarbazide blockades were completely overcome by prolonged (3 hour) exposure to Schiff reagent.

Routine performic-acid-Schiff reactions were done on a number of tissues, and negative reactions are recorded for glycogen, epithelial mucins, cartilage matrix, collagen of cornea, sclera and areolar and capsular tissues generally, basement membranes, bone matrix, yeasts, bacteria, actinomyces, hypophyseal colloid and cell granules, and chromaffin. Ocular melanin reacts negatively and is partly to completely bleached. Ocular lens capsule and Descemet's membrane color light pink to colorless. Certain of the lipofuscin pigments react positively, at least in part, notably that of the human ovary. Myelin colors pink to red.

The detailed technic of the performic-acid-Schiff method as currently used is presented:

Performic acid reagent: To 9.2 g. 90% formic acid (8 ml.) add 33.7 g. 30% hydrogen peroxide (31 ml.) and 0.4 g. concentrated sulfuric acid (0.22 ml.). The temperature should be 25°C. or lower. About 4.7% performic acid is formed within two hours and the solution deteriorates after a few hours. Hence the reagent should be made up fresh for each batch of sections.

Fixation and sectioning method must be such as to preserve the materials which are the specific object of examination. For ceroid, aqueous formaldehyde solutions and paraffin embedding are satisfactory. The recommended schedule is: (1) Bring sections to water in the usual manner, treating as usual to remove mercurial precipitates, if necessary. (2) Oxidize sections for 1 to 2 hours, best about 90 minutes, in freshly prepared performic acid reagent. (3) Wash 10 minutes in running water or in several changes of tap water. (4) Immerse in Schiff reagent for 10 minutes. (5) Wash in 3 changes of 0.5% sodium metabisulfite ($Na_2S_2O_5$) or bisulfite ($NaHSO_3$) of 1,2, and 2 minutes duration. (6) Wash 10 minutes in running water or in several changes of tap water. (7) Stain 6 minutes in Weigert's acid iron chloride hematoxylin, which should be at least 1 hour old and not over a week old. (8) Wash 4 minutes in running water (or several changes). (9) Counterstain 1 minute in saturated aqueous picric acid (C.I. No. 7) solution. (10) Dehydrate directly in 2 changes each of 95% and absolute alcohol, clear in 1 change of absolute alcohol and

xylene (50–50) and 2 changes of pure xylene, and mount in polystyrene or other suitable synthetic resin. Sites of reaction are red to purple.

After some "softer" fixations nuclei also color red, but this source of confusion is eliminated by use of an alum or iron hematoxylin counterstain which colors nuclei blue or black. This nuclear staining is an ordinary Feulgen nucleal reaction, since it is induced equally by formic (or acetic) acid mixtures of the same concentration as in the performic (and peracetic) reagents, when hydrogen peroxide is omitted.

Since permanganates also oxidize ethylenic compounds (Karrer, 1950) it was considered desirable to attempt to confirm the results of the performic acid experiments by using potassium permanganate. I have previously reported (1951a) that this reagent produced a Schiff-positive compound from ceroid (Casella reaction) and that the action of permanganate on most Casella-positive substances can be prevented by the same acetylation procedure as for the HIO_4-Schiff reaction. Experiment showed that a 24-hour acetylation weakened but did not prevent this $KMnO_4$-Schiff reaction, that a 1-hour bromination also weakened but did not abolish the reaction, and that application of acetylation followed by bromination before the oxidation step completely prevented the red coloration of ceroid.

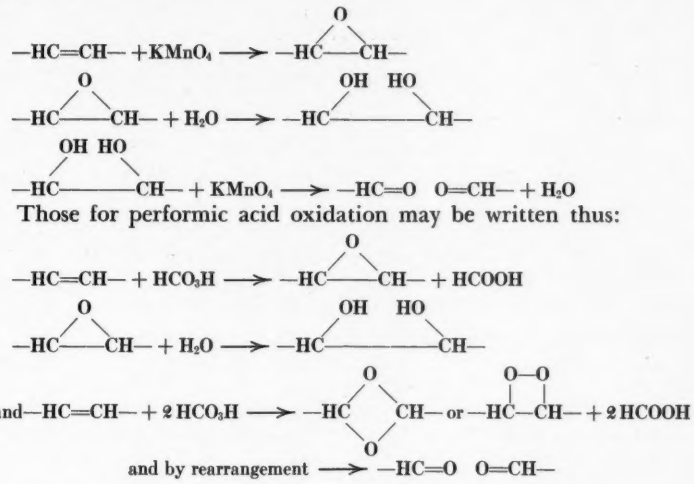
Discussion

It would appear that ceroid possesses both ethylenic groups and 1,2-glycols. This conclusion is supported by the positive periodic-acid-Schiff reaction after bromination and the positive performic-acid-Schiff reaction after acetylation, and by the blocking of the periodic acid and performic-acid-Schiff reactions respectively by acetylation and by bromination.

Under our histochemical conditions with a large excess of oxidant, the product of performic acid oxidation of ethylenic groups appears to be at least in part aldehyde, rather than 1,2-glycol exclusively, as it seems to be when stoichiometric proportions or an excess of unsaturated compound is used with organic peracids. At the same time it appears that performic acid does not attack 1,2-glycols to form aldehydes, since the larger proportion of periodic-acid-Schiff positive substances do not form demonstrable aldehyde with performic acid. The positive Schiff reaction and the positive periodic-acid-Schiff reactions after very prolonged treatment with performic acid indicate that neither aldehyde nor 1,2-glycol is destroyed by performic acid oxidation.

Since 1,2-glycol is not oxidized to aldehyde by performic acid, it would appear that the oxidation of ethylene to aldehyde must avoid

the 1,2-glycol step. The oxidation of C = C groups to aldehyde with $KMnO_4$ is written thus:



Those for performic acid oxidation may be written thus:

Ethylenic peroxides readily decompose to aldehydes or ketones (Rieche, 1931; Machu, 1937).

The question remains whether epoxide groups are attacked by performic or by periodic acid. Since performic oxidation is completely blocked by bromination, and epoxides should offer no open valences, it appears unlikely that the epoxides are attacked by performic acid. Since epoxides should hydrolyze readily in acid solution to 1,2-glycol, periodic acid oxidation appears the more probable answer to the question posed above.

While this paper was in press, the report of Wolman (1950) came to my attention. Wolman stated that various unsaturated fats in spot tests, as well as in tissues, give positive red purple reactions on 10-minute exposure to Schiff reagent after oxidation with chromic acid, with permanganate, and with periodic acid, and that prior acetylation does not abolish this reaction. Wolman used the acetylation of McManus and Cason. I have previously (1951a) pointed out the inadequacy of this technic, and since Wolman records a positive periodic-acid-Schiff reaction of renal basement membranes after his acetylation, it is evident that the acetylation was inadequate. This structure has been among the more easily acetylated in my hands, as well as in those of McManus.

Wolman did not practice bromination. For these reasons his findings with chromic acid and with permanganate are only suggestive

as regards demonstration of ethylenic linkages, and those with periodic acid are misleading.

Conclusions

It is believed that the presence of ethylenic groupings in ceroid has been demonstrated by performic acid oxidation. Under histochemical conditions, the large excess of performic acid present carries the oxidation directly from ethylene state to aldehyde. Performic acid does not form aldehyde from 1,2-glycols under the histochemical conditions used.

The demonstration of ethylenic groups in ceroid as well as 1,2-glycols and presumably epoxides as well, supports the thesis that ceroid may be derived from highly unsaturated dietary fats.

I am indebted especially to H. J. Burtner for assistance in this study.

References

Edwards, J. E., and White, J. 1941. Pathologic changes, with special reference to pigmentation and classification of hepatic tumors in rats fed p-dimethylanimoazobenzene (butter yellow). J. Nat. Canc. Inst., **2,** 157–83.

Elftman, H., Kaunitz, H., and Slanetz, C. A. 1949. Histochemistry of uterine pigment in vitamin E-deficient rats. Ann. N. Y. Acad. Sci., **52,** 72–9.

Endicott, K. M. 1944. Similarity of the acid fast pigment ceroid and oxidized unsaturated fat. Arch. Path., **37,** 49–53.

Endicott, K. M., Daft, F. S., and Sebrell, W. H. 1944. Dietary cirrhosis without ceroid in rats. Proc. Soc. Exp. Biol. Med., **57,** 330–1.

Endicott, K. M., and Lillie, R. D. 1944. Ceroid, the pigment of dietary cirrhosis of rats. Its characteristics and its differentiation from hemofuscin. Amer. J. Path., **20,** 149–53.

Findley, T. W., Swern, D., and Scanlan, J. T. 1945. Epoxidation of unsaturated fatty materials with peracetic acid in glacial acetic acid solution. J. Amer. Chem. Soc., **67,** 412–14.

Greenspan, F. P. 1946. The convenient preparation of peracids. J. Amer. Chem. Soc., **68,** 907–8.

Györgi, P., and Goldblatt, H. 1942. Observations on the conditions of dietary hepatic injury (necrosis, cirrhosis) in rats. J. Exp. Med., **75,** 355–68.

Hickinbottom, W. J. 1941. Reactions of organic compounds. Longmans, Green and Co., London, New York and Chicago.

Karrer, P. 1950. Organic Chemistry, *4th Ed.* Elsevier Publishing Co., New York, Amsterdam, London & Brussels.

Lee, C. S. 1950. Histochemical studies of the ceroid pigment of rats and mice and its relation to necrosis. J. Nat. Canc. Inst. **11,** 339–49.

Lillie, R. D. 1948. Histopathologic Technic. Blakiston Co., Philadelphia and London.

Lillie, R. D. 1951a. Histochemical comparison of the Casella, Bauer and periodic acid oxidation-Schiff leucofuchsin technics. Stain Techn., **26,** 123–36.

Lillie, R. D. 1951b. Histochemical studies on the retina. (In press, Anat. Rec.)

Lillie, R. D., Ashburn, L. L., Sebrell, W. H., Daft, F. S., and Lowry, J. V. 1942. Histogenesis and repair of the hepatic cirrhosis in rats produced on low protein diets and preventable with choline. Pub. Health Rep., **57,** 502–8.

Lillie, R. D., Daft, F. S., and Sebrell, W. H. 1941. Cirrhosis of the liver in

rats on a deficient diet and the effects of alcohol. Pub. Health Rep., **56,** 1255–8.

MACHU, W. 1937. Das Wasserstoffperoxyd und die Perverbindungen. J. Springer, Vienna.

RIECHE, A. 1931. Alkylperoxyde und Ozonide. Th. Steinkopf, Dresden and Leipsig.

SCANLAN, J. T., and SWERN, D. 1940. Action of lead tetra-acetate upon hydroxylated fat acids and related compounds. J. Amer. Chem. Soc., **62,** 2305–9; 2309–11.

SMITH, M. I., LILLIE, R. D., and STOHLMAN, E. F. 1943. The toxicity and histopathology of some azo compounds as influenced by dietary protein. Pub. Health Rep., **58,** 304–17.

SWERN, D., BILLEN, G. N., FINDLEY, T. W., and SCANLAN, J. T. 1945. Hydroxylation of monounsaturated fatty materials with hydrogen peroxide. J. Amer. Chem. Soc., **67,** 1786–9.

WOLMAN, M. 1950. Staining of lipids by the periodic acid Schiff reaction. Proc. Soc. Exp. Biol. & Med., **75,** 583–5.

A DIRECT SILVER METHOD FOR THE GOLGI APPARATUS

HERBERT ELFTMAN[1], *Department of Anatomy, College of Physicians and Surgeons, Columbia University*

Received for publication Aug. 4, 1951

ABSTRACT.—**Direct immersion of fresh tissue in a solution of silver in formalin at pH 4, followed by development in hydroquinone-formalin, results in consistent silvering of the Golgi apparatus. The time required depends on the penetration of the tissue, two hours for each step being adequate for routine purposes. Proper general fixation of the tissue is enhanced by returning it to a fixative for the customary periods of time. A weak solution of iron alum is suggested as a convenient method for reducing the intensity of the silver image in sections, when that is desired. Replacing the silver image with gold allows it to survive more drastic subsequent treatment, such as periodic acid oxidation.**

The application of silver to tissues of the barn owl, preserved in a mixture containing osmium, led Camillo Golgi in 1898 to the discovery of the cytoplasmic component which has since been known by his name. For more than half a century silver and osmium have vied with each other as reagents by means of which permanent preparations of the Golgi apparatus could be achieved, in order to facilitate the study of its relation to cellular function.

Both osmium and silver methods have earned the reputation of being capricious. Many investigators have attempted to make them more uniformly reliable. The efforts in this direction for the silver methods have emphasized variations in the fixative employed preceding the application of silver. To the formalin which is common to all methods, various adjuvants have been added. Among these are the arsenious acid of Golgi (1908), uranyl nitrate of Cajal (1912), cobalt nitrate of Da Fano (1920) and cadmium chloride of Aoyama (1929). By careful adjustment of time of fixation, of subsequent immersion in silver nitrate, and of development, some good results may be achieved by each of these methods.

Each of these modifiers of Golgi's original technic assumed correctly that the fickleness of the method was due to the treatment employed before immersion in the silver solution. They made the further assumption, however, that this pretreatment was necessary for the success of the procedure. Experiments which we have conducted show that the Golgi substance will react with the silver re-

[1]With the technical assistance of Gloria Collins.

agent directly, provided the tissue is fresh. The function of the adjuvants added to the formalin fixative by previous investigators is to protect the reactivity of the Golgi substance rather than to enhance it. Under these circumstances there is no purpose in attempting to unravel the Gordian knot of pretreatment before immersion in silver, since the pretreatment may be cut out entirely.

Although the details of the direct silver method are subject to many variations, its essential features will be illustrated by citing one practical routine.

(1) Immerse fresh tissue in 2% $AgNO_3$ in 15% formalin for 2 hours.
(2) Develop in 2% hydroquinone in 15% formalin for 2 hours.
(3) Return to an appropriate fixative, such as 15% formalin, until general fixation is complete.
(4) Run up, embed in paraffin and section as usual.

When examination of the tissue is urgent, this schedule may be accelerated. We have taken out small pieces of tissue in the morning and studied them, in paraffin section, in the middle of the afternoon. Such an accelerated schedule does not degrade the Golgi apparatus but obviously does not provide sufficient time for proper tissue fixation.

The Silver Solution

The essential step in the direct silver method for the Golgi apparatus is to allow the silver ions to have access to the fresh tissue in a formalin solution of proper acidity. Some tissues are more sensitive than others to variations in hydrogen ion concentration but at pH 4 the appearance of the Golgi apparatus will parallel that obtained by the older osmium and silver methods. The employment of buffers affords the usual insurance of uniformity in technic but the limited solubility of most silver salts restricts the concentration of silver ion when buffers are used.

The concentration of the silver solution may be varied over a wide range, the rate of penetration increasing with concentration. For routine use it has been convenient to employ 2% silver nitrate for 2 hours or 0.7% silver acetate in acetate buffer for 5 hours. Prolonged immersion results in denser Golgi images, which may easily be bleached by subsequent treatment, but it also influences the reaction of the tissue to counterstains. When the latter factor is not important, the tissue may be left in the silver solution overnight or longer.

The customary injunction that silver procedures should be carried out in the dark can easily be over-emphasized when they are performed on blocks of tissue. The production of a layer of metallic silver over the surface of the tissue screens the interior just as adequately as a layer of black paper surrounding the vial.

The formalin used for this procedure must not be contaminated with chloride or carbonate ions since they will precipitate the silver. Commercial formalin, diluted with 1½ to 6 volumes of water, fulfills these requirements and also provides a congenial degree of acidity for the silver reaction. It is also possible to add other reagents, such as picric acid, care being taken to adjust the pH.

Developer

The choice of developers is limited by the pH at which the reaction takes place since it is advisable to change the environment of the tissue as little as possible in going from the first step to the second. The combination of formalin and hydroquinone, utilized so extensively by Cajal in his silver methods, fulfills these requirements. The use of 2% hydroquinone in formalin of the same strength as that used in the silver solution provides adequate penetration of the average block of tissue in two hours. With the developer, as with the silver solution, it is the rate of penetration rather than the rate of the reaction which determines the time that is necessary. Lengthening the time incurs no serious disadvantage, overnight development frequently being a convenience. Among the developers which can be substituted for hydroquinone are pyrogallol and metol.

Subsequent Treatment of Tissue

Unless the tissue has been left for more than the minimum time necessary in the silver solution and in the developer, it is advisable to return it to a fixing solution. The purpose of doing this is not to affect the Golgi image but to permit the completion of fixation of other tissue elements. Although formalin of the same concentration as that chosen for the first two steps is an obvious first choice, other fixatives may be employed, provided that they are compatible with the silvered tissue.

When fixation has been satisfactorily accomplished, the tissue is prepared for embedding by the usual routine. Paraffin embedding allows sections to be cut at the usual thicknesses, 2μ and 5μ being most generally useful.

Selective Bleaching and Intensification

It is possible to adjust the silver solution and the developer so that exactly the desired degree of silvering of the Golgi apparatus is accomplished. The same result can be achieved with equal precision, but with greater ease, by applying to the slide a solution which will either intensify or partially bleach the image. Since intensification, the methods for which may be found in any photographic handbook, may lead to extraneous precipitates of silver, there is a practical advantage in working with tissue in which the silver images are too dense rather than too light.

The process of decreasing the density of the silver is known as reduction in photography but we shall call it bleaching in order to avoid chemical ambiguity. It consists essentially of the oxidation of a portion of the metallic silver by a solution which will not precipitate insoluble silver salts. Bearing in mind the oxidation potential of silver and the low concentration of silver in the section, it is possible to devise innumerable bleaching methods without calling for chemicals not present in every laboratory. The use of permanganate is unnecessarily complicated, since the reduction of the manganese yields manganese dioxide, which must be removed. Our current favorite is iron alum. In a 0.1% solution the proper

FIG. 1. (2000×). The Golgi apparatus of the epididymis of the mouse. 2% silver nitrate in 25% formalin for 2 hours; 2% hydroquinone in 25% formalin for 2 hours; embedded in paraffin; sectioned at 2 μ; counterstained with methylene blue.

amount of bleaching occurs in a matter of minutes, allowing appropriate determination of the endpoint under the microscope. Rinsing in water stops the reaction and the ferrous iron which results disappears without special attention.

If bleaching or counterstaining is not contemplated, it is possible to remove the paraffin and mount the slide without running it down to water. Although such a procedure is proper when curiosity must be satisfied immediately, the retention in the tissue of some unreduced silver may lead to eventual darkening. Although most of the silver ions diffuse out of the tissue when the fixation time is prolonged, they have a more satisfactory opportunity for escape from the cut

section. For this reason it is advisable to run the slides down to water even when they are not to be subjected to additional procedures. The use of hypo for this rinse is not recommended. The virtues of this reagent in photography stem chiefly from its ability to form soluble complexes with silver halides; it has additional properties which are far from simple. Since the chloride ions of tissue are found chiefly in the intercellular fluid and in the erythrocytes, silver halides do not confuse the cellular picture, while the other actions of hypo may.

Gold Replacement

The ease with which the silver image is bleached seriously interferes with many subsequent procedures. In order to utilize a variety of technics on the tissue, it is necessary that the silver be replaced by a metal which is less easily oxidized. Of the metals which could be used for this purpose, gold has long given universal satisfaction.

In order for this replacement to occur, the slide is placed in a solution containing ionic gold. The concentration of the gold, usually gold chloride, and the time allowed do not need delicate adjustment unless it is desired to stop the reaction before it is complete. Since gold chloride is very soluble, its excess dissolves in water; the possibility that some of the silver may remain as silver chloride if only water is employed makes rinsing in hypo rational at this point.

The use of gold for the replacement of silver is a time-honored practice in histological technic under the term "gold toning". While this term accurately describes the function of the process in photography and in some histological technics, the use of gold replacement advocated here is not for tone but for survival.

Counterstaining

The form which the Golgi apparatus possesses in the prepared section and the relation which it has to the nucleus can be readily observed without counterstaining. Further correlation between these features and other properties of the tissue is greatly facilitated when tissue constituents can be differentiated by staining. Methylene blue and phloxine are satisfactory general stains and aniline blue, either with or without phosphotungstic acid, is useful for some purposes.

A more generous variety of methods may be used when the silver has been replaced by gold. A gold image will survive periodic acid oxidation and immersion in the Schiff reagent as well as most of the other reagents used in histochemical research.

In the interpretation of the results of staining, the same caution must be exercised here as with all fixed and stained tissue. The state of the proteins after fixation in silver nitrate, formalin and hydroquinone under relatively acid conditions is obviously different from

that produced by neutral formalin or other fixatives. The staining proclivities of the tissue must be interpreted in terms of this previous history rather than by direct comparison with tissues fixed by other methods. After the direct silver method, periodic acid can oxidize some tissue constituents which remain Schiff negative after similar oxidation of formalin-fixed tissue. Since silver is an oxidant, such results should not be unexpected.

Interpretation of the Silver Image

All that is dark with silver in these preparations does not belong to the Golgi apparatus. The Golgi material, like other cell constituents, is defined by a constellation of characteristics, not the least of which is its response to changes in the functional state of the cell.

The shape and the position of the Golgi apparatus after this or any other method is obviously the resultant of its normal disposition in the living cell and the changes induced by the reagents which have acted upon it. No matter how drastically the treatment may have altered the state of aggregation of the material, the fact that identical treatment results in vastly different types of aggregation of the Golgi material in different cells provides us with a powerful tool for investigation. With the method of preparation kept constant, variations in the silvered Golgi image may not directly reflect all of the details of its morphology of the moment but it certainly casts a convincing shadow of the changing capacities of this material to react with an unchanging reagent.

REFERENCES

Aoyama, F. 1929. Eine Modifikation der Cajalschen Methode zur Darstellung der Golgischen Binnennetz Apparate. Zeit. wiss. Mikr., **46,** 489–91.

Cajal (Ramon y Cajal), S. 1912. Formula de fijación para la demonstración fácil del aparato reticular de Golgi. Trab. Labor. Invest. Biol. Univ. Madrid, **10,** 209–20.

Da Fano, C. 1920a. Method for the demonstration of Golgi's internal apparatus. J. Physiol., **53,** xcii–xciv.

——— 1920b. Method for the demonstration of the Golgi apparatus in nervous and other tissues. J. Roy. Micr. Soc., **40,** 157–61.

Golgi, C. 1898. Sur la structure des cellules nerveuses. Arch. Ital. Biol., **30,** 60–71.

——— 1908. Une méthode pour la prompte et facile démonstration de l'appareil rèticulaire interne des cellules nerveuses. Arch. Ital. Biol., **49,** 269–74.

CHROMATOGRAPHY AND BIOLOGICAL STAINS

I. Paper Chromatography as a Means of Determining Purity and Dye Content of Sudans III and IV.[1]

John F. Christman and Robert H. Trubey, *Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge, Louisiana*

Received for publication Aug. 13, 1951

Abstract.—**By paper chromatographic technics, using iso-octane as the solvent, we have been able to analyze commercial Sudans III and IV for the numerous colored components present. This technic is invaluable in the qualitative analysis of the dyes studied, and, in addition, a quantitative estimation can be accomplished by a serial dilution method described. It would appear that there is a difference between the Sudan III or Sudan IV concentration found by this method and the total dye content found by the chemical reduction method. A detailed description of the procedure and a discussion of the results are included.**

Since the advent of World War I there have been great strides made in the manufacture and staining efficiency of biological stains. Dye content, however, is still usually measured by the non-specific chemical reduction method which measures the concentration of the azo-group in the dye mixture. Recently research has been carried out on the spectrophotometric characteristics of some of the biological stains (Conn and Stotz, 1946; Stotz *et al.*, 1950). These analyses, however, depend on the gross characteristics of the dyestuff; there remains no technic which will adequately assay a sample of stain for impurities.

Since paper partition chromatography has been introduced (Consden, Gordon and Martin, 1944) a method is now available which will rapidly analyze a given sample of material for the chemical entities although the exact nature of the process has yet to be elucidated. A wide variety of mixtures have been separated by this technic, including amino acids, vitamins, carbohydrates, organic acids and plant pigments (see the excellent symposium published by the Biochemical Society, 1949). No successful separations of biological stains have been reported. Paper chromatography is par-

[1]The authors wish to express their appreciation to the U. S. Atomic Energy Commission [Contract No. AT-(40-1)-1095] and RG-2975 of the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service for partial support of this work. Appreciation is also due the National Aniline Division, Allied Chemical and Dye Corporation, New York, for supplies of the dye used.

ticularly well adapted for these compounds since the visual analysis is apparently adequate following development.

Procedure

The technic used was quite similar to that of Horne and Pollard (1948). In this procedure the mobile solvent system ascends a hanging paper strip through capillarity. The immobile phase is air-saturated with a volatile solvent. This technic is readily adaptable to a wide variety of solvent systems, and it is considerably simpler to manipulate than the earlier described descending method (Consden *et al., op. cit.*). The paper used was Whatman No. 1 filter paper, cut into strips 13 cm. by 44 cm. The solvent used for the separation of the Sudan dyes was commercial grade synthetic iso-octane (2,2,4-trimethylpentane) as the mobile and stationary solvents. The iso-octane was added to both the outer chamber and center well of the chamber.

The chambers were 8½″ by 18″ Pyrex brand cylindrical jars, equipped with a 15 cm. by 2.5 cm. Pyrex brand petri dish as the center well for the mobile solvent. A hanger for the strips was constructed as follows: a hole was drilled into the center of a plate glass plate, 12″ square by ¼″ with a ⅜″ diamond drill; a ⅛″ hole was drilled through a $^{5}/_{16}$″ × 1½″ machine screw approximately $^{3}/_{16}$″ from the end of the screw; the screw was fastened to the plate through two tight-fitting rubber gaskets with a nut, and a $^{3}/_{16}$″ wire was placed through the small hole and held in place by a second nut. The strips can then be hung from the wire. To prevent corrosion, the metal parts should be cadmium or chromium plated.

A sample of the dye to be chromatogramed was dissolved in a suitable solvent such as alcohol or petroleum ether, and 0.05 ml. was added slowly to the reference line (about 3 cm. from the bottom of the strip) so that the colored area did not exceed 0.5 cm. in diameter.

The chamber is equilibrated with iso-octane for at least 24 hours in a constant temperature room (25°C.) with 100 ml. in the outside chamber and 150 ml. in the center well. The prepared strip is then fastened to the wire hanger with stainless steel clips and the assembly lowered into the chamber so that about 8 mm. of the lower end of the strip is immersed in the solvent. The glass plate acts as a cover, and when greased with a silicone base grease makes an air tight system.

As the solvent front advances one can readily follow the separation of the colored components. After the front has advanced approximately 30 cm. (12 hours at 25°C.) the strip is removed, and the front ratio (R_f) values calculated in the usual fashion.

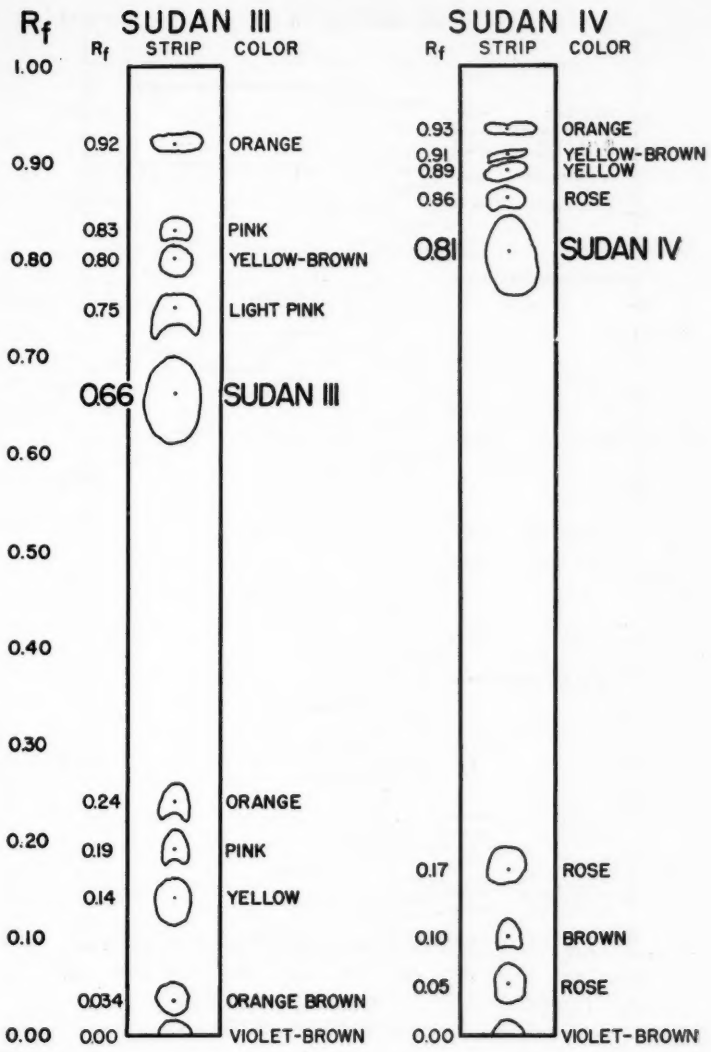


FIGURE 1:

Diagramatic representation of paper chromatograms of Sudans III and IV. Sample 0.05 ml. of suspension 0.25 g. of dye to 25 ml. of 95% ethyl alcohol. Solvent system: Isooctane. Development 12 hours at 25°C.

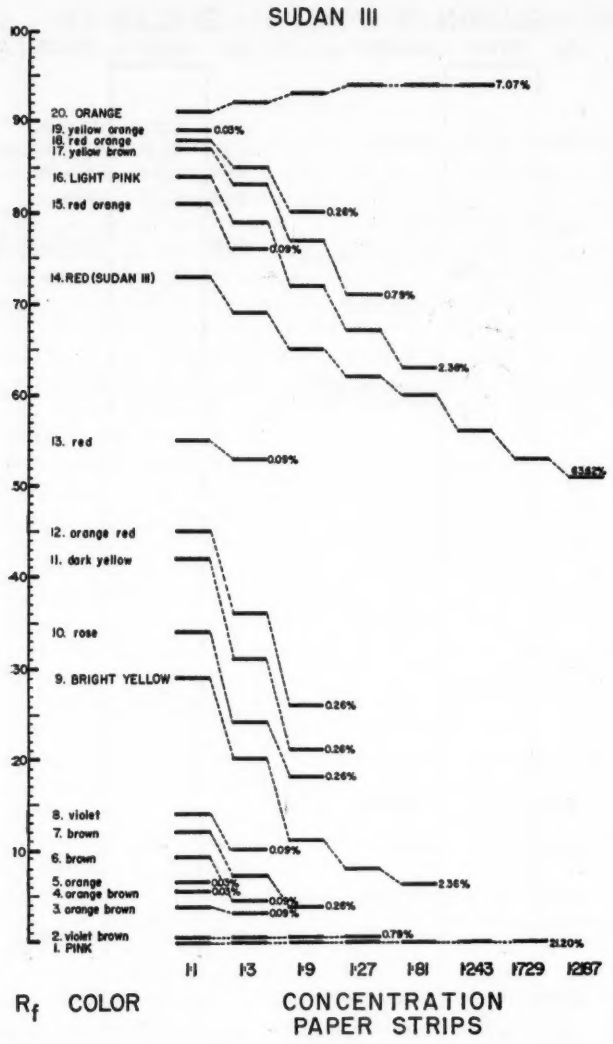


FIGURE 2:

Line diagram of series of paper chromatograms of 1:3 serial dilutions of Sudan III. Initial concentration of suspension: 3.0 g. in 25 ml. of 95% ethyl alcohol. Strips contain 0.05 ml. at each concentration. Solvent system: Isooctane. Development 12 hours at 25°C. Dye colors given in capital letters refer to major components.

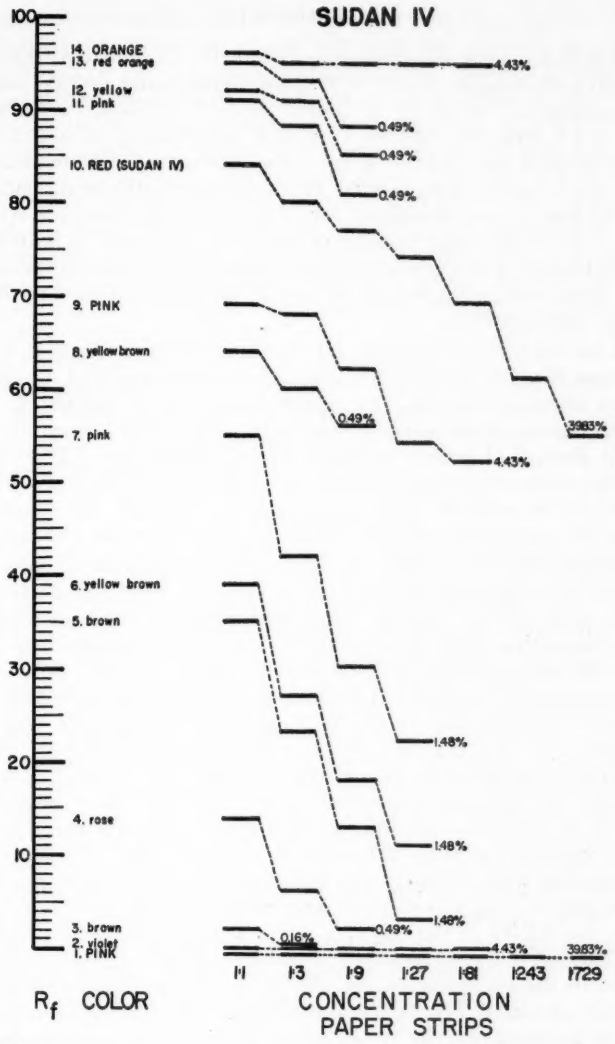


FIGURE 3:

Line diagram of series of paper chromatograms of 1:3 dilutions of Sudan IV. Initial concentration of suspension: 3.0 g. in 25 ml. of 95% ethyl alcohol. Strips contain 0.05 ml. at each concentration. Solvent system: Isooctane. Development 12 hours at 25°C. Dye colors given in capital letters refer to major components.

Results

A 0.25 g. sample of commercial Sudan III (National Aniline Division, Certification No. NY 6, 90% dye content) and a 0.25 g. sample of commercial Sudan IV (National Aniline Division, Certification No. NZ 9, 86% dye content) were each suspended in 25 ml. of 95% ethyl alcohol, heated to 60°C. for a few minutes to facilitate solution, and the solutions were filtered. Strips were prepared using 0.05 ml. of the filtrates and developed for 12 hours in the iso-octane system. Fig. 1 shows the large number of colored components in both stain preparations. The fractionations are quite distinct, and reproducible R_f values were obtained on numerous attempts within 0.05 R_f at these concentrations.

A second sample of Sudan III (National Aniline Division, Certification No. NY 10, 92% dye content) was prepared and developed in an identical manner. The colored areas were as follows: R_f 0.00 violet brown, 0.038 orange brown, 0.11 yellow, 0.63 Sudan III, 0.72 light pink, 0.77 yellow brown, 0.80 red orange and 0.92 orange. With minor exceptions the two preparations are identical with respect to the colored components present, although the concentrations of a particular substance might be different in the two samples.

When 0.005 ml. of this solution was chromatogramed, only four stains were apparent, the R_f 0.00 violet brown, 0.63 Sudan III, 0.70 light pink, and 0.91 orange. These data indicate that a quantitative approximation might be obtained on the concentrations of the various colored materials in a particular sample of stain. One could chromatogram a sample of stain in serial dilutions, determine the lowest concentration at which a particular component was still visible, and calculate the relative percentages with respect to the original concentration of material. To test this method, a 3.0 g. sample of Sudan III, (Certification No. NY 10) was suspended in 25 ml. of 95% ethyl alcohol and heated to boiling for two minutes. The solution was allowed to cool slightly and settle; the slurry was withdrawn with a pipette and 1:3 serial dilutions (1 part dye solution plus two parts of solvent) were made until the final concentration was 1:2187 of the original. Strips were prepared each containing 0.05 ml. of the various concentrations and after 12 hours development in the iso-octane system, the strips were removed and the R_f values calculated. Fig. 2 is a graphical plot of R_f versus concentration. It can be noted that the R_f value for a particular colored component (connected by dotted lines) varies with a change in concentration, and further that the rate of variation (measured graphically by the slope of the connecting lines) changes with position on the chromatogram. This phenomenon has been established previously for column chromatography (LeRosen, 1947).

Although this technic depends on the assumptions that the eye is

equally sensitive to identical concentrations of various pigments and that equal concentrations of the pigments will separate as single components, it is obvious, nevertheless, that certain components are present in much greater concentration than others. If one computes the relative concentration of these materials on the basis of the lowest concentration that is still recognizable, relative percentages can be calculated for each component (as presented in Fig. 2).

By this technic it would appear that only 63.6% of the total dye content is Sudan III. Even though this value is a quantitative approximation, it is probably much closer to the true value with regard to the Sudan III concentration, than that value obtained by the chemical reduction method, since the latter not only includes the amount of reduction by Sudan III, but also all of the other diazo groups present in the other pigments in the mixture.

This same serial dilution procedure was followed for Sudan IV (Certification No. NZ 9, 86% dye content) with the results plotted in Fig. 3. By similar calculation one finds that the Sudan IV is present in apparently only 39.3% of the total dye.

Discussion

It has been possible by paper chromatographic technics to demonstrate the presence of multiple colored constituents in commercial samples of Sudans III and IV. This method of stain analysis is a rapid, extremely sensitive system which can be employed without the need for expensive equipment. It has been employed extensively in our laboratories in analyses of Sudan III fractionations.

It is of interest to note that of the two different samples of Sudan III tested both showed practically the same components.

A method of quantitative approximation is presented to determine the relative concentrations of the various colored substances in the commercial samples. Although the accuracy of this method is limited by certain visual errors, it nevertheless presents an estimation of the true concentration of substances present in distinct contrast to the chemical reduction method which no doubt includes not only the dyestuff sought, but numerous other contaminating compounds.

The values obtained for the concentration of Sudan III and Sudan IV present in the commercial samples is distinctly lower that that reported by the chemical reduction method.

REFERENCES

Conn, H. J., and Stotz, E. 1946. Comparative adsorption readings obtained with spectrophotometers of various types. Stain Techn., **21**, 49–54.

Consden, R., Gordon, A. H., and Martin, A. J. P. 1944. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J., **38**, 224–232.

Horne, R. E., and Pollard, A. L. 1948. The identification of streptomycin on paper strip chromatograms. J. Bact., **55**, 231–234.

LeRosen, A. L. 1947. The rate of movement of a chromatographic zone as a function of column position, initial concentration and initial volume. J. Amer. Chem. Soc., **69**, 87–90.

Stotz, E., Conn, H. J., Knapp, F., and Emery, A. J. 1950. Spectrophotometric characteristics and assay of biological stains. I and II. Stain Techn., **25**, 57–68.

Williams, R. T., *ed.* 1949. Partition Chromatography; Biochemical Society Symposia No. 3. The University Press, Cambridge, England.

DIFFERENTIATION OF TWO TYPES OF BASOPHILS IN THE ADENOHYPOPHYSIS OF THE RAT AND THE MOUSE

NICHOLAS S. HALMI, *Department of Anatomy, State University of Iowa, Iowa City*

Received for publication Aug. 13, 1951

ABSTRACT.—**Pituitaries are fixed for 24 hr. in Bouin's fluid containing 0.5% trichloroacetic acid instead of 5% acetic acid, or in a mixture of 9 parts SUSA and 1 part saturated aqueous solution of picric acid. They are embedded in paraffin and horizontal sections are cut at 3-4 μ. The staining method consists of 3 phases: (a) immersion in aldehyde-fuchsin for the selective demonstration of the beta cell granules, (b) staining of the nuclei with Ehrlich's hematoxylin and (c) a rapid one-step counterstain with light green and orange G dissolved in a phosphotungstic-acetic acid mixture for the differentiation of the acidophilic and the delta cell granules.**

"Kresazan", the combined "kresofuchsin" (or resorcin-fuchsin) and "azan" method of Romeis (1940) for the differentiation of 2 classes of adenohypophysial basophils (the beta cells, whose granules stain with resorcin-fuchsin, and the delta cells, whose granulation lacks this affinity and takes up aniline blue or its analogues), did not become a routine procedure for the staining of the hypophysis for the following reasons: (a) the original kresofuchsin preparation is no longer available and resorcin-fuchsin is cumbersome to prepare, (b) the time of staining with the dye is extremely variable (1–24 hr.), (c) doubt was cast on the validity of the distinction between the two cell types by the work of other authors (Brolin, 1945, Goldberg and Chaikoff, 1950), who had difficulties in attempting to duplicate Romeis' results. The discovery of the technically much more satisfactory elastic tissue stain, aldehyde-fuchsin, and the demonstration of its affinity towards the same hypophysial basophil granules which take up resorcin-fuchsin (Gomori, 1950) led to a confirmation of Romeis' contentions as to the existence of two tinctorially distinct types of basophils in the adenohypophysis of several species. Subsequent observations on the rat hypophysis (Halmi, 1950, 1951) provided further evidence of morphological and very striking histophysiological differences between the beta and the delta basophils in the rodent. The staining technic applied in these studies was a combination of aldehyde-fuchsin and azan, with light green substituted for aniline blue. This procedure, although less cumbersome

[1]National Aniline, Cert. No. NF 57, used by author.

and more reliable than "kresazan," still is not simple enough to be recommended as a routine method. However, it can be modified to become both simpler and faster. Since the pituitary of the rat (and the mouse) does not appear to contain acidophils which have a special affinity for azocarmine (carmine cells), alum hematoxylin can be used as a nuclear stain, and the staining time can be considerably further reduced by adding the mordant to the light-green:orange G counterstain. Thus simplified, the staining technic is reliable and permits the staining of up to 50 slides per day. It should, in the author's opinion, replace routine methods which do not differentiate between the physiologically distinct beta and delta basophils in histological studies on the pituitary of the rat, the mouse and other species which do not possess carmine acidophils.

Procedure

Fixation. The use of an acid fixative is preferable, because it destroys at least the majority of the mitochondria, which could be mistaken for acidophilic granules, and also because it makes the vacuolated cells more conspicuous by coagulating the vacuolar contents. Furthermore, with the use of the ideal fixative there should be little or no background staining with aldehyde-fuchsin; the secretory granules should be stained specifically and brilliantly; the nuclei should be clearly defined; the sections should display a high degree of transparency, since cell counts are performed under oil immersion. Although several fixatives have been tried, only modifications of Bouin's fluid and Heidenhain's SUSA mixture were found to meet most, but not all, of these requirements.

Bouin's fluid containing 5% acetic acid has several disadvantages: it frequently causes hemolysis; it leads to a coarse precipitation of the nongranular cytoplasm which may simulate secretory granules; and a tendency towards overstaining with light green is often encountered after its use. The reduction of the acetic acid content to 2% partially eliminates these flaws, but replacing the acetic acid altogether with 0.5% trichloroacetic acid was found to be even more satisfactory.

SUSA seems to penetrate faster and fix more evenly than the fixatives of the Bouin type. It also gives excellent nuclear definition and transparency. However, after its use there is more background staining with aldehyde-fuchsin than after Bouin's fluid, the dispersion of the beta granules is finer (which makes the identification of poorly granulated beta cells more difficult), and also the staining of the delta granules with light green often lacks brilliancy. These flaws can be largely remedied if 1 part of a saturated aqueous solution of picric acid is added to 9 parts of SUSA, although in this case the staining of the acidophilic granules with orange G may become somewhat duller.

The hypophyses should be fixed in either the modified Bouin's fluid or the SUSA-picric mixture for 24 hr. and subsequently washed in tap water for several hours. The tissues can be stored in 70% or 95% alcohol.

Embedding and sectioning. Our previous practice of double embedding in celloidin and paraffin has been abandoned in favor of paraffin only (melting point 56–58°C), since it was found that celloidin, even if removed completely with ether-alcohol, might abolish the selective staining of the beta granules with aldehyde-fuchsin. Sections should be cut in the horizontal plane at 3 or 4 μ.

Staining procedure.

1. Run deparaffinized sections through descending alcohols to Lugol's solution. Leave them in the latter for 30 minutes.
2. Remove iodine in 5% aqueous sodium thiosulfate (2 minutes).
3. Rinse slides thoroughly in distilled water.
4. Stain in aldehyde-fuchsin. Make up the following solution 2–3 days in advance:

   60% alcohol .....100 ml.
   Basic fuchsin[1] ..... 0.5 g.
   Paraldehyde .....1 ml.
   Concentrated HCl.....1.5 ml.

   The solution turns purple in 24 hr. It becomes ripe in 3 days at 20°C. and proportionately faster (down to 2 days) at higher temperatures. If a ripe solution is used, the staining time varies between 2 and 10 minutes. Four or 5 days after the preparation of the mixture, the staining time begins to increase rapidly and further aging abolishes the selectivity of the aldehyde-fuchsin for the beta granules completely. Since overstaining has to be avoided, it is advisable to check the slides periodically under the microscope (after rinsing them in 95% alcohol) and to interrupt the staining when the beta cells stand out clearly in dark purple against a colorless or faintly lilac background.
5. Rinse slides in 2 changes of 95% alcohol and leave them in the third change for 5–10 minutes.
6. Rinse in 70% alcohol and distilled water.
7. Stain in Ehrlich's hematoxylin (3–4 minutes).
8. Rinse in distilled water and differentiate in 0.5% HCl-alcohol (3–4 dips).
9. Blue in running tap water (5–10 minutes).
10. Counterstain in the following solution (45 seconds):

    Distilled water .....100 ml.
    Light green SF yellowish[2] .....0.2 g.
    Orange G[2] .....1.0 g.

[2]Dyes used by author were National Aniline products: Light green, NL–12 or 13; Orange G, NO–13; Chromotrope 2R, batch #13511.

Chromotrope 2 R[2] .....0.5 g. (may be omitted)
Phosphotungstic acid .....0.5 g.
Glacial acetic aicd .....1 ml.

The solution keeps indefinitely.

11. Rinse briefly in 0.2% acetic acid and 95% alcohol.
12. Two changes of absolute alcohol (2 minutes each).
13. Blot slides carefully.
14. Two changes of xylene (2 and 5 minutes, respectively).
15. Mount in clarite.

Results

The granulation of the beta cells is stained selectively a dark purple shade with aldehyde-fuchsin. If the tissues were fixed in formol-sublimate or a sublimate-picric-formol mixture, the cells of the pars intermedia and the Herring bodies of the neural lobe are also heavily tinged with the dye. With the use of the recommended Bouin or SUSA modifications, the affinity of the latter structures for aldehyde-fuchsin is less constant or altogether absent. The delta cell granules are stained green with the light green and the acidophilic granules varying shades of orange with the orange G. The nuclear chromatin is purplish brown to reddish brown. The nucleoli are tinged bright red with the chromotrope 2 R. The nongranular cytoplasm is grayish green or unstained. The coagulated contents of cytoplasmic vacuoles stain with orange G.

ACKNOWLEDGMENT

The author is indebted to Dr. George Gomori for many valuable suggestions.

REFERENCES

BROLIN, S. E. 1945. A study of the structural and hormonal reactions of the pituitary body of rats exposed to cold. Acta Anat., *Suppl. III.*, 1–165.

GOLDBERG, R. C., and CHAIKOFF, I. L. 1950. The cytological changes that occur in the anterior pituitary gland of rats injected with various doses of I^{131} and their significance in the estimation of thyroid function. Endocrinology, **46,** 91–104.

GOMORI, G. 1950. Aldehyde-fuchsin: a new stain for elastic tissue. Amer. J. Clin. Path., **20,** 665–6.

HALMI, N. S. 1950. Two types of basophils in the anterior pituitary of the rat and their respective cytophysiological significance. Endocrinology, **47,** 289–99.

HALMI, N. S. 1951. Further observations on two types of basophil cells in the anterior pituitary. Anat. Rec., **109,** 300.

ROMEIS, B. 1940. Die Hypophyse. *In* v. Möllendorff's Handbuch der mikroskopischen Anatomie des Menschen. Julius Springer, Berlin, **6,** Part 3, 79–80 and 591.